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WHEY PROTEIN STABILIZATION AND LACTOSE HYDROLYSIS FOR THE PRODUCTION OF WHEY-BASED BEVERAGES

by

© VICTOR MANUEL BERNAL OLIVERA

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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Supervisor

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Date Edmonton, 30/8/84

Abstract

The major objectives of this research were related to two technical problems found when considering the manufacture of a non-fermented beverage from lactose-hydrolyzed whey: 1) to prevent the formation of a protein sediment in the drink as a consequence of the heat treatment applied to these products; and 2) to evaluate several commercial lactase preparations for the hydrolysis of lactose in cottage cheese whey and to select the best enzyme among those preparations to be used in the manufacture of the drink.

In the first part of the study, several factors affecting the thermal stability of the proteins present in whey were studied over 2.5-6.5 pH range using Differential Scanning Calorimetry (DSC) and heat precipitation studies. The highest denaturation temperature for an acid whey protein concentrate prepared by ultrafiltration was 88°C at pH 3.5, while for pure β-lactoglobulin(β-lg) the highest denaturation temperature, obtained at pH 3.5, was 81.9°C. Presence of milk sugars (lactose, glucose and galactose) appeared to increase the resistance of β-lg against thermal denaturation. In bovine serum albumin preparations, denaturation temperature varied with fatty acid contents. The importance of calcium for the thermal stability of α-lactalbumin was proven by a 20-22°C decrease in the denaturation temperature upon the addition of 0.1 M EDTA. Heating of whey at 95°C for 5 minutes above pH 3.8-3.9 produced extensive protein precipitation. When the same heat

treatment was applied below pH range 3.7, protein precipitation was prevented.

In a preliminary hydrolysis experiment, the effectiveness of lactose hydrolysis by a soluble β-galactosidase from *Kluyveromyces lactis* was compared in skim milk, KOH-treated cottage cheese whey, KOH-treated ultrafiltration permeate, and model lactose solutions. Of the three possible industrial substrates, the highest β-galactosidase activity was observed in whey, followed by ultrafiltration permeate and milk. The addition of β-lactoglobulin, bovine serum albumin and ovalbumin to buffered lactose solutions had no effect on the activity of the enzyme. The hydrolysis of lactose seemed to be dependent upon the amount of potassium present. The K_m of the enzyme for lactose in 0.025 M KH_2PO_4 was 76.9 mM. Comparison between NaOH and KOH-treated whey confirmed the suitability of potassium as neutralizing agent when this enzyme is used.

In the main hydrolysis study, six commercial β-galactosidase (E.C. 3.2.1.23) preparations were compared for the hydrolysis of lactose in cottage cheese whey. The comparison was based on parameters obtained from the kinetic characteristics of the enzymes in lactose solutions (Michaelis-Menten constants, apparent turnover numbers, initial reaction rates and integrated equations derived from the Michaelis-Menten expression), and on experimental progress curves for the hydrolysis of lactose in whey. The conversion data were used to predict optimum enzyme

dosage/hydrolysis time combinations to attain a fixed degree of lactose conversion. The K_m values for lactose varied from 24.0 to 150.2 mM. The lactose hydrolysis efficiency in cottage cheese whey was different for each enzyme. The use of acid β-galactosidases did not impart any undesirable sensory characteristics to a grapefruit flavoured prototype product developed in the laboratory.

Sensory evaluation experiments (triangle tests) showed no significant difference in bitterness (1.0% confidence level) between a protoype product sweetened with sucrose and several drinks made from whey treated with any of the three best acid β-galactosidases to an 80% lactose hydrolysis level. Based on the results obtained and including an economic evaluation of the commercial preparations, the most suitable β-galactosidase preparations available for use in cottage cheese whey were identified.

This study demonstrated that it should be possible to manufacture a lactose hydrolyzed whey beverage without protein sedimentation problems and with acceptable organoleptic characteristics. The cost of the enzyme required to carried out the lactose hydrolysis would be only about 3 or 4 cents per litre of whey, under the conditions described in this work.

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1. INTRODUCTION.

1.1 Whey and whey beverages.

The production of cottage cheese in Canada in 1982 was 30,984,000 kg (Agriculture Canada, 1983). Of this total, 4,958,000 kg were produced in Alberta. As a byproduct of the manufacture of cottage cheese, 44,622,000 kg of acid whey were produced in the province (Agriculture Canada, 1983).

Wherever cheese is produced, the utilization of whey presents many interesting opportunities. While the high nutritional and functional value of the whey proteins is well known, the major component of whey, lactose, is considered to be less valuable. Development of new uses or improvement of the existing functional properties of lactose would open up new possibilities for the use of this byproduct in the food and dairy industries (Jelen, 1983).

The modification of whey by enzymatic hydrolysis of lactose offers new ways of utilization for this cheesemaking byproduct. In places where the whey is used as animal feed, higher unit consumption by pigs and cattle could be expected from hydrolysed whey (Burguess and Shaw, 1983).

Lactose hydrolysed milk is suitable for the preparation of concentrated milk products. Ice cream, baby foods, cheddar and cottage cheeses, and yogurt are some of the products that have been considered (O'Leary and Woychik, 1976; Woychik and Holsinger, 1977; Guy, 1980; Ismail *et al*. 1983).

Hydrolysed whey syrups could be used as sweeteners in dairy and confectionary products (Coton, 1979). The utilization of hydrolysed whey in the baking industry could replace whole milk, skim milk and whey powders. The utilization of whey as a fermentation substrate has been studied extensively (Kosikowski, 1979). Single cell protein, whey wines and methane are some of the possible products of this hydrolysed whey fermentation (Shukla, 1975; Woychik and Holsinger, 1977; Gawel and Kosikowski, 1978; Chojnowski *et al.*, 1978; Kosikowski, 1979). Perhaps the most viable alternative would be the production of ethanol from whey for the manufacture of vinegar and drinks (Coton, 1979).

The development of nutritional beverages is a very promising area for the successful utilization of hydrolyzed whey. Small cheese manufacturers would be especially benefitted, since the production of flavoured drinks from fluid whey is a relatively simple process which avoids expensive steps such as drying, condensing or fractioning of the whey. In particular, acid whey is highly compatible with acid fruit flavours. The unique composition of these products gives them an enormous potential as highly nutritious and electrolyte balanced drinks, ideal for sportsmen, children and the ill (Kosikowski, 1968; Holsinger *et al.*, 1977; Szakaly *et al.*, 1978; Kosikowski, 1979). In the manufacture of nutritious nonfermented whey-based soft drinks, the common practice has been to blend a whey powder or a whey concentrate with frozen, concentrated or powdered fruit juices. A soft drink based on 50% hydrolysed whey and flavoured with tropical fruit or coconut extract was developed in Switzerland (Fresnel and Moore, 1978). A beverage prepared by mixing clarified whey with 40-50% fruit juice concentrate from oranges and passion fruit, sodium cyclamate and saccharin, was patented in West Germany (Schutz, 1982). The preparation of an orange flavoured shelf-stable athletic-type beverage made from lactose hydrolysed and deproteinated whey base was described recently by Crippen and Jeon (1984). In January 1983, a whey based drink appeared in the Edmonton area. Whey protein and hydrolysed lactose are added to a 50% juice fluid containing passion fruit, orange, lemon, grapefruit and pineapple juice (Landers, 1982). The product is distributed by Canada Safeway Ltd. under license of Imperial Juice Inc.. The drink was developed by Arla Company of Sweden and has been also introduced in Sweden, France, the U.S. and Japan.

1.2 Enzymes in the dairy industry.

Probably the most important application of enzyme technology in the dairy industry is the use of rennet in the production of cheese. Recently the use of β-galactosidase in the hydrolysis of lactose in milk and milk products has found commercial applications, becoming the second major use of enzyme technology in the dairy industry (Fox, 1980).

Lactase (β-D-galactoside galacto-hydrolase ; E.C. 3.2.1.23) is the common name for intestinal β-galactosidase.

Lactase catalyzes the hydrolysis of lactose, acting upon its 1-4 glycosidic linkage to release glucose and galactose. This lactose splitting enzyme is widely distributed in nature; it can be found in plants, fungi, yeasts, bacteria, and the intestine of young mammals (Nijpels, 1982).

Lactose (4-(β-D-galactopyranosyl)-D-glucopyranose) is a disaccharide found in the milk of female mammals. After water, it is the most abundant component of milk (Shukla, 1975). This milk sugar has low solubility and sweetness, and it cannot be absorbed directly from the intestine (Nijpels, 1982). In contrast, its constituent monosaccharides glucose and galactose have a higher sweetening power, are three to four times more soluble than lactose, and are easily absorbed from the intestine.

Although the potential for β-galactosidase in dairy processing has been recognized for a long time (Woychik and Holsinger, 1977), it was not until the development of commercial enzyme preparations from microbial sources in the last 15-20 years that large scale application of this enzyme became a reality.

Even if the 1983 world-wide enzyme sales ($400 million U.S.) were below the $1 billion predicted for 1985 in the mid 70's, this volume of sales is steadily growing at a 6.7% annual rate (Catalano, 1984). Within the food industry, the market for the enzymes used in the manufacture of high fructose corn syrup (amylase, amylose glucosidase and glucose isomerase) was worth $100 million last year, while the rennin market was worth approximately $80 million (Catalano, 1984)

Three companies control as much as 80% of the enzyme market: Novo Industri from Denmark, Gist-Brocades from Holland, and Miles Laboratories from the US. However, as many as 30 other companies now participate in the enzyme business (Catalano, 1984). Most of the small companies cannot afford the high costs associated with the development of new products, so they are offering low-priced generic versions of top-selling enzymes. At least 10 enzyme companies offered β-galactosidase preparations in North America in 1984 (IFT, 1984).

By means of a β-galactosidase treatment, milk and other dairy products can be made suitable for a large proportion of the world's population. Many adults or individuals from certain ethnic or racial groups cannot digest lactose due to the intestinal lactase deficiency and can therefore only take lactose-containing dairy products in very small quantities.

1.3 Objectives of the Research Work.

The present work was part of a major collaborative project related to the development of whey-based beverages. However, the aim of this work was not the methodical product development of a marketable whey drink. Rather, the major objectives of this study were focused on two technical problems found in the manufacture of a lactose hydrolyzed acid whey beverage: 1) the prevention of the protein precipitation caused by the heat processing of the lactose hydrolyzed product, and 2) the selection of the best soluble enzyme available for the hydrolysis of lactose in cottage cheese whey. A third objective was to evaluate any possible effect that the use of this enzyme could have on the sensory characteristics of prototype whey drinks produced in the laboratory.

The tendency of the whey proteins to precipitate, particularly under the influence of heat, is an important problem generally faced by whey beverages. Acid and heat stability of the proteins are two very important criteria when it is desired to produce an acid beverage from whey, since pasteurization or mild sterilization are necessary to make these beverages shelf-stable. Although some acid-stable whey protein concentrates designed for the fortification of citrus and other acid-based beverages are commercially available (Anonymous, 1981a; Andres, 1982), protein sedimentation may still be a problem. A visible sediment layer was reported to appear during storage in several whey drinks recently introduced in Europe (Jelen and Bucheim, 1984). Basic studies on the denaturation, aggregation and precipitation of whey proteins during heat treatment at low pH were necessary to understand the conditions under which these phenomena could be controlled.

In the second part of the study several β-galactosidase preparations were evaluated for their effectiveness to hydrolyze lactose in cottage cheese whey. The enzymatic hydrolysis of lactose would permit the production of a whey-based beverage - with approximately one quarter of the protein and practically all of the sugar and minerals present in milk - that could be consumed by lactose intolerant people.

To select the best soluble β-galactosidase available, six commercial enzyme preparations were compared on the basis of their basic kinetic parameters, their performance in cottage cheese whey, the sensory evaluation of the hydrolyzed products, and the cost. The use of a free β-galactosidase in soluble form was considered to be the right choice for the hydrolysis of lactose in whey, particularly when taking into account the size of the cheesemaking industry in Alberta.

In the third part of this project, prototype lactose-hydrolyzed whey drinks were tested by two sensory evaluation panels to establish if the use of the selected β-galactosidase preparations could have any detrimental effects on the flavour of these drinks, and to determine the pattern of preference of this drink against a commercial whey drink already in the market.

2. LITERATURE REVIEW.

2.1 Milk intolerance.

Milk and dairy products have been part of the human diet for many years, and are now recognized as one of the most nutritive and versatile basic food groups. However, consumption of milk and dairy products could create health problems in some cases. Milk proteins are responsible for milk allergy, the most common of the food allergies. The symptoms produced by this anaphylactic reaction are not severe, however, and the importance of this illness is widely overlooked (Speer, 1978). Current research at the Institute for Dairy Research in Kiel, West Germany, seems to indicate that the formation of a hard casein clot in the stomach, due to the acid coagulation of milk, may be the reason for certain type of milk rejection (Meisel and Hagemeister, 1984). This hard casein clot problem could gain importance in the future, and will probably be the object of further studies by research groups in other parts of the world. Nevertheless, the most well known problem related to the ingestion of milk is the so called lactose intolerance, due to lactose malabsorption in the intestine.

Milk contains only one carbohydrate, lactose. Immediately after birth the ability of all mammals to digest lactose is very important. Since no disaccharides can be absorbed as such in the intestine, lactose has to be hydrolysed into glucose and galactose before the energy of these sugars can be used by the organism. The hydrolysis of lactose is performed by the enzyme β-galactosidase, which is normally found in the mucosa of the small intestine (Nijpels, 1981).

The activity of lactase in the fetus gradually increases from the third month of the pregnancy throughout gestation and reaches a maximum at full term. During the weaning period, mammals are fed only with milk. Lactase activity is the highest in the perinatal period, and slowly diminishes as milk is gradually replaced by other foods. By adulthood, lactase activity drops to about one tenth of its maximum level (Lutkic and Votava, 1982). The main decline in activity occurs between three and five years of age. The disappearance of lactase seems to be less drastic in the individuals consuming milk from domestic animals (Anonymous, undated, a).

It is believed that lactase deficiency results from a combination of socio-cultural, nutritional, geographical and genetic factors: milk consumption habits, low vitamin D synthesis in areas with low incidence of natural UV irradiation and congenital lactase deficiency, and others. Lactase activity seems to be independent of lactose intake (Nijpels, 1982). Some causes of temporary lack of lactase activity are premature birth, gross undernourishment and intestinal tract surgery.

Lactose intolerance is defined by the clinical effects which occur in a person with proven lactose malabsorption following oral ingestion of 50g of lactose mixed in water, after overnight fasting (Paige and Bayless, 1981). The more common clinical effects of lactose intolerance after lactose intake include abdominal pains, diarrhea, flatulence and other gastrointestinal disturbances accompanied by a very slow rise in blood sugar level. However, lactose intolerance does not imply milk intolerance. It has been estimated that the incidence of milk intolerance among persons showing lactose intolerance will be about 25%, after drinking one glass of milk (Nijpels, 1981).

Appart from the unpleasant symptoms described above, lactose malabsorption and milk intolerance will have some undesirable nutritional effects like the irritation of the intestines and water losses from the body into the colon due to the production of lactic acid and carbon dioxide by the intestinal microflora. A number of studies have proven that all the previously mentioned symptoms do not occur when lactose is replaced by a mixture of glucose and galactose (Anonymous, undated, a).

2.2 Functional properties of hydrolysed lactose.

Apart from the benefits that the use of β-galactosidase would bring to the people suffering from milk intolerance, there are also certain technological advantages that make the hydrolysis of lactose interesting.

There are several functional differences between a pure lactose solution and a mixture of glucose, galactose and lactose. The main differences between these two solutions are given in table 2.1. Lactose is much less soluble than hydrolysed lactose. The hydrolysis makes it possible to increase the total soluble sugars in a concentrated syrup. Crystallization in the hydrolysed lactose solution is not a problem below 70% total solids (Shah and Nickerson, 1978b). The viscosity of the hydrolysed syrup is relatively low compared to that of the pure lactose solution. Shah and Nickerson (1978c) showed that 100% hydrolysis was not necessary since maximum sweetness was achieved with 70-90% hydrolysis, due to the synergestic sweetening effect of the three sugars. The limited fermentability of the lactose solution is increased when glucose is produced by hydrolysis.

2.3 Characteristics of β-galactosidase.

This enzyme (E.C.3.2.1.23) catalyzes the hydrolysis of lactose in the β-D-galactoside linkage, liberating one mole of D-glucose and one mole of D-galactose (Burguess and Shaw, 1983). The different sources from which this enzyme has been isolated are listed in table 2.2. Enzyme preparations of commercial value for the dairy and food industries are isolated from yeasts and fungi (Nijpels, 1982).

The primary role of lactase in nature is probably the hydrolysis of lactose that occurs as the first step in the conversion of lactose from the milk of female mammals into energy. However, this enzyme also has the capacity to

Table 2.1.- Comparison of the functional properties of lactose and 80% hydrolyzed lactose.

	Lactose	80% hydrolyzed Lactose
Solubility at 40°C[1] (g anhydrous sugar/100g sol.)	24	63
Viscosity at 40°C[1] and 60% solids (cP)	29	18
Sweetness(relative to sucrose)[2]	20-40	65-85

[1] From Shah and Nickerson, 1978a.

[2] From Anonymous, undated, a.

Table 2.2.- Some sources of β-galactosidase; adapted from Finocchiaro *et al*. (1980).

- **Plants**
  - Peach
  - Apricot
  - Almond
  - Alfalfa seed
- **Animal organs**
  - Intestine
- **Yeast**
  - *Kluyveromyces lactis*
  - *Kluyveromyces fragilis*
  - *Candida pseudotropicalis*
- **Bacteria**
  - *Escherichia coli*
  - *Streptococcus lactis*
  - *Streptococcus thermophilus*
  - *Lactobacillus bulgaricus*
  - *Lactobacillus helveticus*
- **Fungi**
  - *Aspergillus niger*
  - *Aspergillus oryzae*
  - *Aspergillus flavus*
  - *Mucor miehei*

hydrolyse other oligo- or poly-saccharides containing D-galactose bound to a β-galactoside (mostly by 1-4 or 1-6 linkage).

2.4 Commercial sources of β-galactosidase.

Commercially available β-galactosidase preparations are usually derived from yeasts (*Kluyveromyces fragilis*, *Kluyveromyces lactis* and *Candida pseudotropicalis*) and fungi (*Aspergillus niger* and *Aspergillus oryzae*). The major difference between the yeast and fungal enzymes is in the optimum conditions for hydrolysis. Yeast enzymes have optimum activity in the pH range 6.0-7.0 and temperatures of 35-40°C. Fungal enzymes have optimum activity in the pH range 4.0-5.0 and temperatures of 50-60°C. The optimum pH of the enzymes has been the main factor determining a particular area of application. The neutral β-galactosidases are best suited for the hydrolysis of lactose in milk and sweet cheese whey and its permeate, while the fungal β-galactosidases are used with the acid whey and permeates (Burguess and Shaw, 1983).

Both yeast and fungal enzymes are inhibited by galactose, one of the products of the hydrolysis. This is the main reason that it is almost impossible to achieve 100% hydrolysis. Product inhibition is particularly important when concentrated substrates are used. The enzyme activity is also influenced by some minerals. Heavy metals (Cu, Zn, Hg) inhibit both types of lactases. Sodium and calcium inhibit the yeast enzymes, while potassium, magnesium and manganese activate them. Since most of these minerals are present in milk and whey, the hydrolysis conditions should be determined for each specific case (Burguess and Shaw, 1983).

2.4.1 Neutral lactases.

Kluyveromyces lactis lactase. This enzyme is available as a purified liquid lactase preparation. The optimum conditions for hydrolysis are near the natural pH of fresh milk, 6.6-6.8 (figure 2.1) Acid whey has to be adjusted to the appropriate pH with a suitable neutralizing agent such as potassium hydroxide. The temperature-activity profile shows an optimum at 35-40°C (figure 2.3). Although the enzyme activity is low at low temperatures (4-6°C), the use of this enzyme has been considered for the treatment of milk during the overnight cold storage period (Nijpels, 1982). The upper temperature limit is set by the denaturation temperature of the enzyme. A short hydrolysis period (3-4 hours) is recommended if the hydrolysis is carried out at 30-40°C, due to possible microbial contamination problems.

Kluyveromyces fragilis lactase. This commercial preparation is obtained by submerged fermentation (Anonymous, 1981b). The differences between the lactases of *K. lactis* and *K. fragilis* are minor. Their optimum conditions of hydrolysis are very similar. The pH and temperature activity profiles

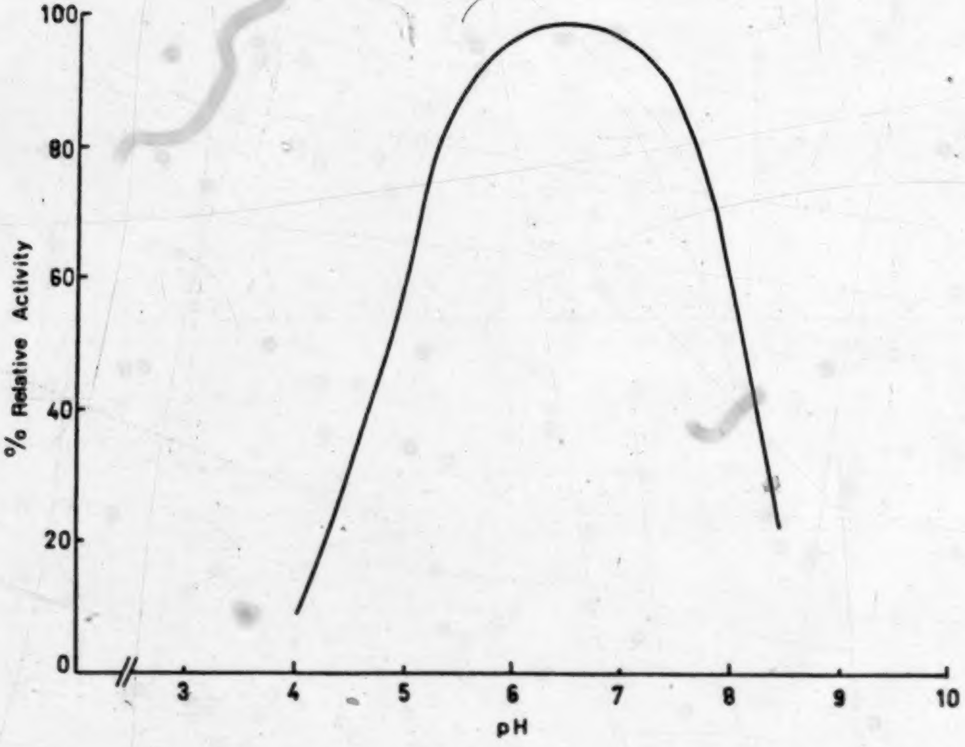

Figure 2.1. Effect of pH on the activity of *Kluyveromyces lactis* lactase(Anonymous, undated, c).

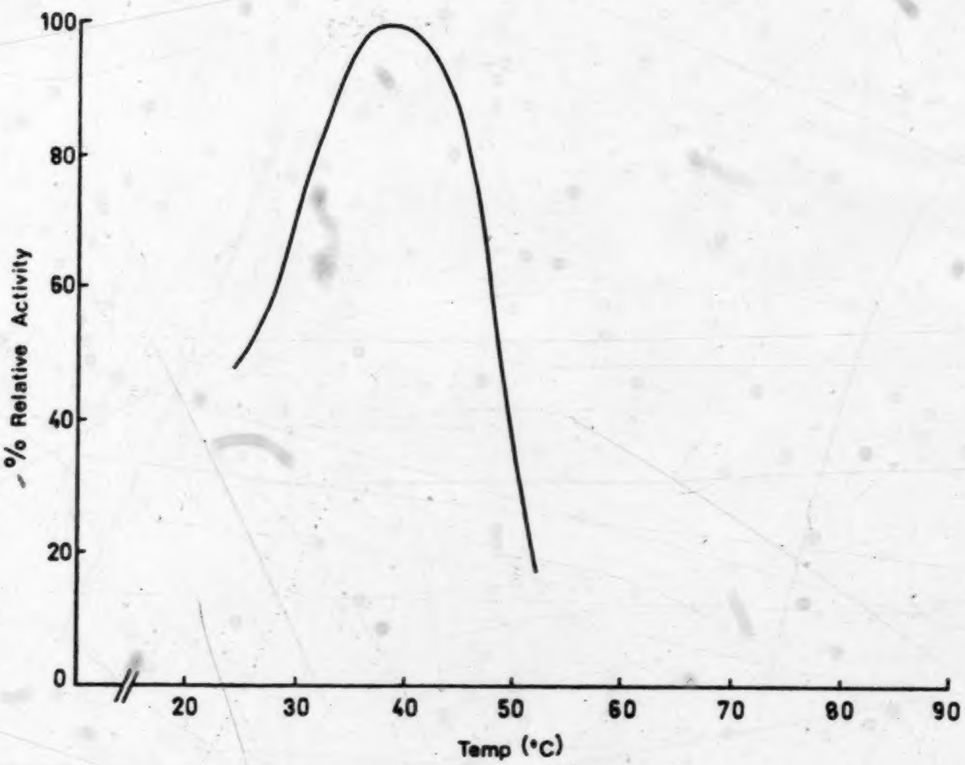

Figure 2.2. Effect of temperature on the activity of *Kluyveromyces lactis* lactase(Anonymous, undated, c).

of the *K. fragilis* lactase are given in figures 2.3 and 2.4.

Candida pseudotropicalis lactase. This β-galactosidase is the most recent neutral lactase introduced in the market. The peaks of activity of this enzyme are between pH 6.2 and 7.0, and between 31 and 40°C (figures 2.5 and 2.6).

2.4.2 Acid lactases.

Aspergillus niger lactase. This enzyme has different optimum conditions in comparison to the yeast lactases. Its activity is the highest at pH values between 3.5 and 4.0 (figure 2.7). An advantage for the use of *A. niger* lactase is the low rate of growth of microorganisms at this pH. The temperature-activity profile is shown in figure 2.8. Although the maximum activity is observed at 60-65°C a temperature of 50°C is recommended for the thermal stability of the enzyme (Nijpels, 1982).

Aspergillus oryzae lactase. The pH and temperature optima for this enzyme are slightly different from those of the *A. niger* lactase. This β-galactosidase has a wide pH-stability range but a narrow optimum between 4.5 and 5.0 (figure 2.9). The temperature of maximum activity is about 55°C (figure 2.10) *A. oryzae* preparations are prefered over *A. niger*, since the product inhibition by galactose is supposed to be less pronounced with the former enzyme type (Sprossler and Plainer, 1983).

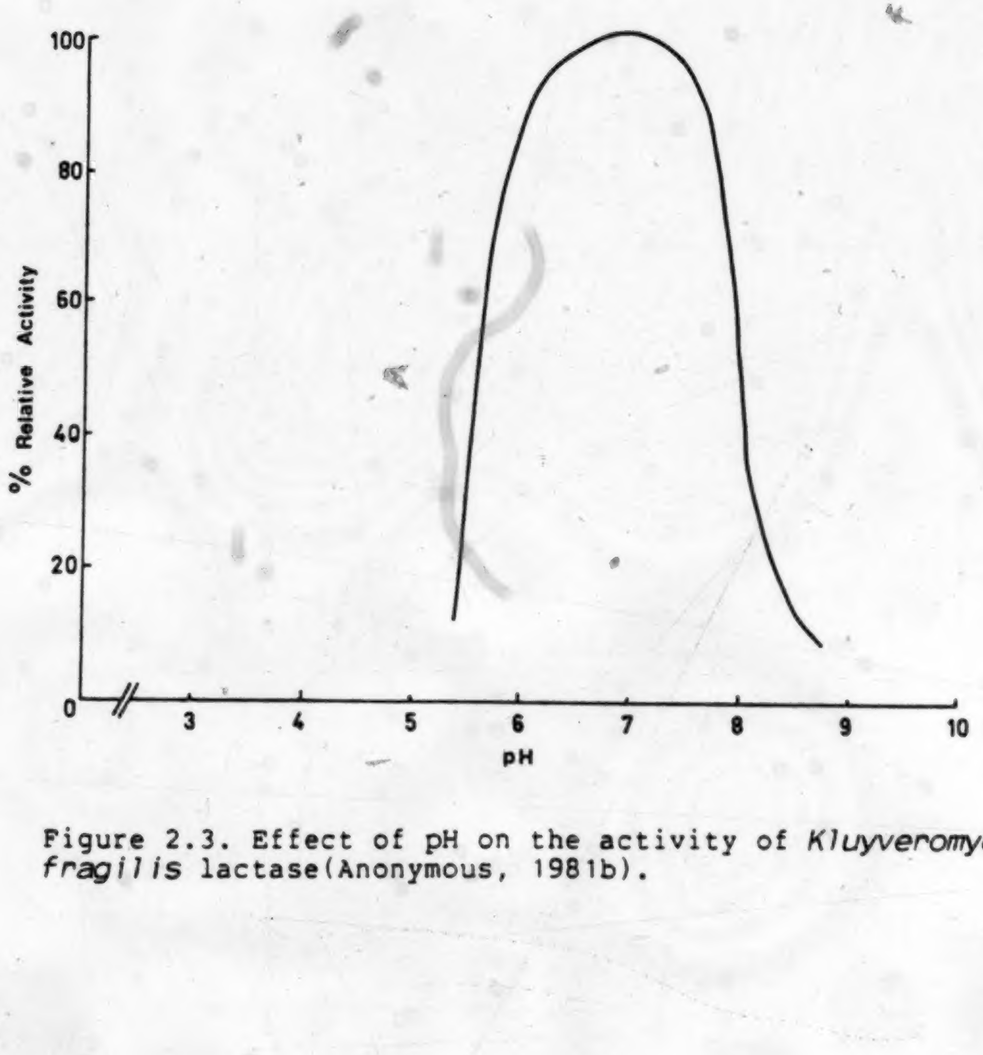

Figure 2.3. Effect of pH on the activity of *Kluyveromyces fragilis* lactase(Anonymous, 1981b).

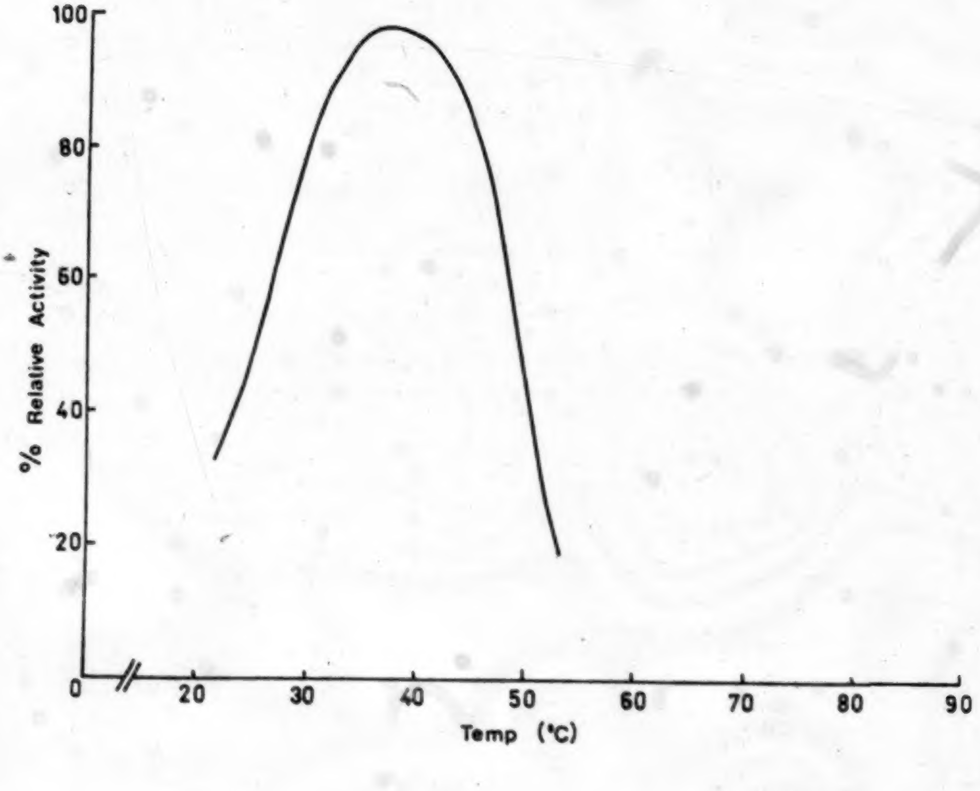

Figure 2.4. Effect of temperature on the activity of *Kluyveromyces fragilis* lactase(Anonymous, 1981b).

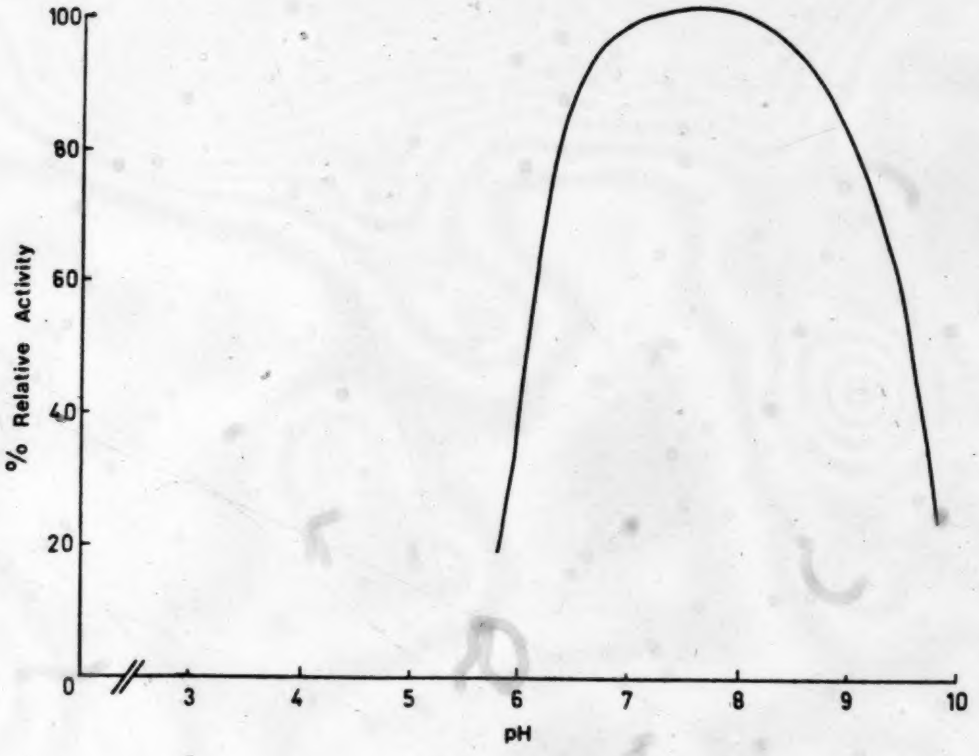

Figure 2.5. Effect of pH on the activity of *Candida pseudotropicalis* lactase(Anonymous, undated, d).

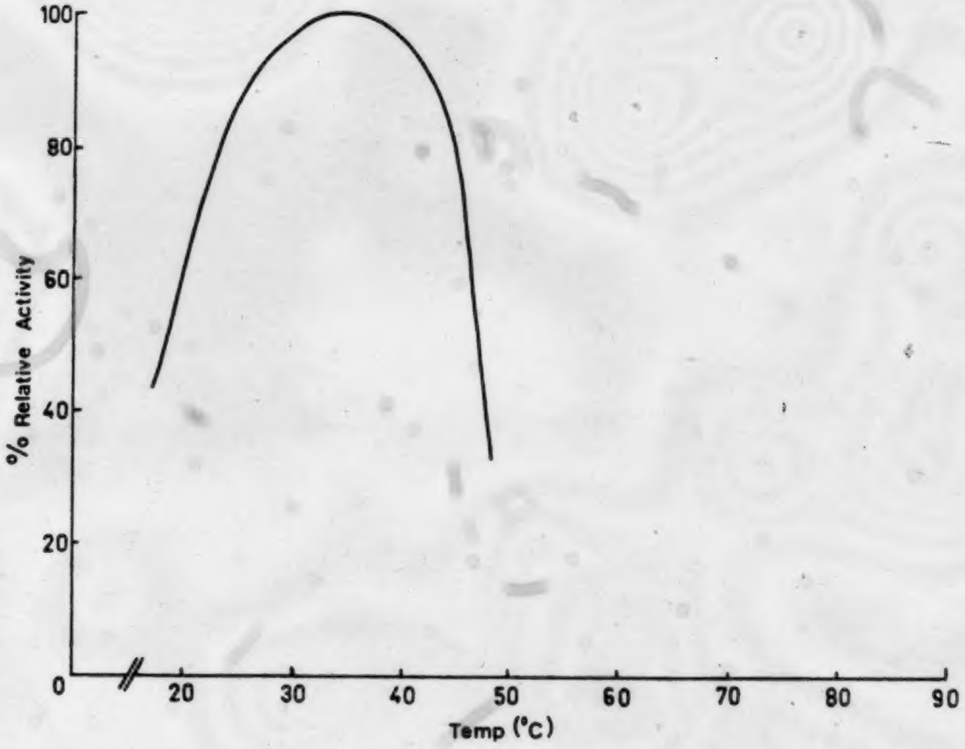

Figure 2.6. Effect of temperature on the activity of *Candida pseudotropicalis* lactase(Anonymous, undated, d).

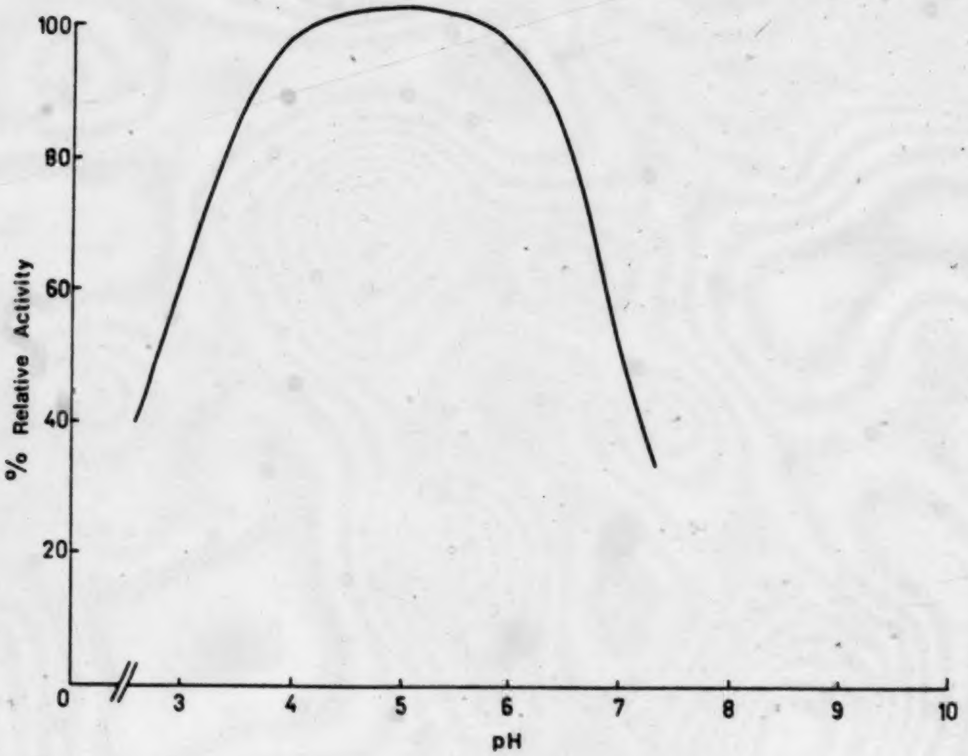

Figure 2.7. Effect of pH on the activity of *Aspergillus niger* lactase(Nijpels, 1981).

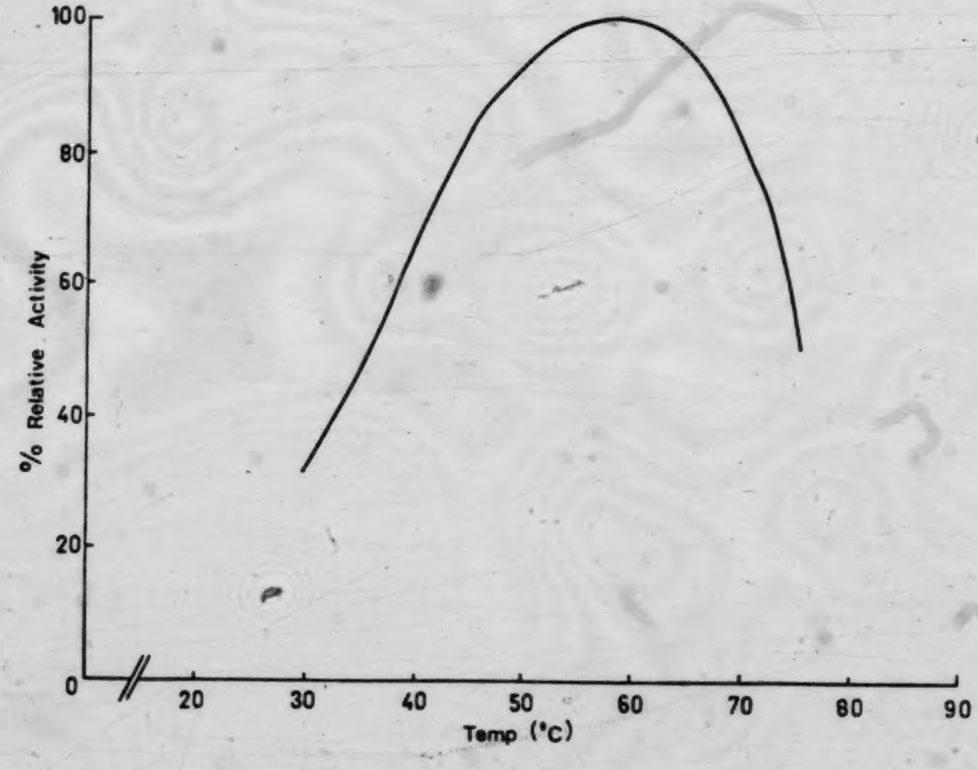

Figure 2.8. Effect of temperature on the activity of *Aspergillus niger* lactase(Nijpels, 1981).

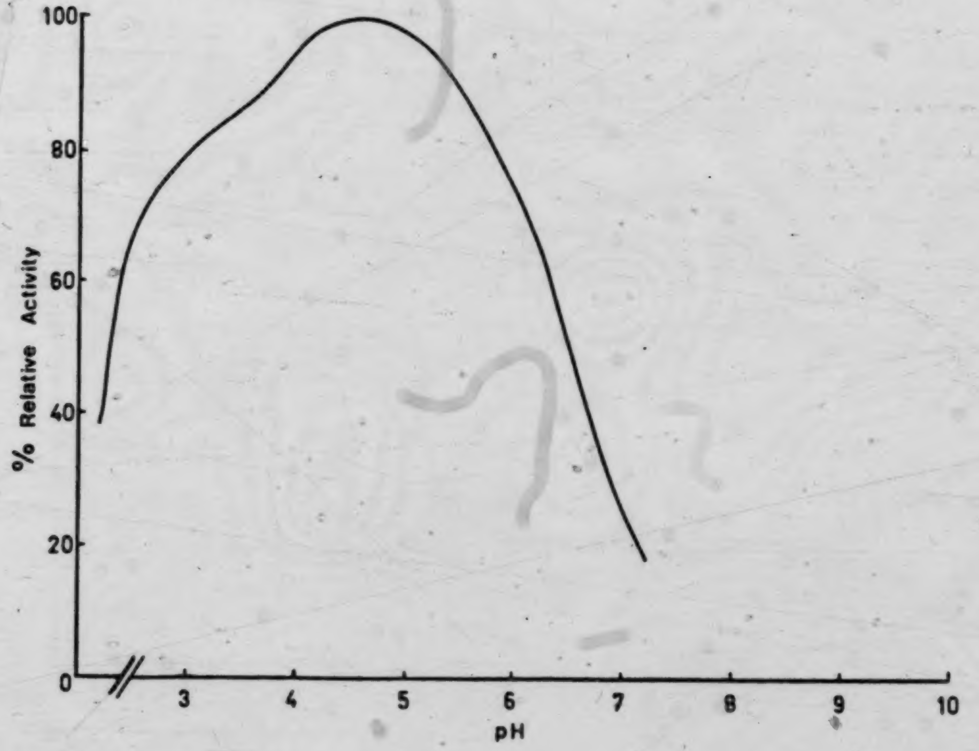

Figure 2.9. Effect of pH on the activity of *Aspergillus oryzae* lactase(Anonymous, 1981d).

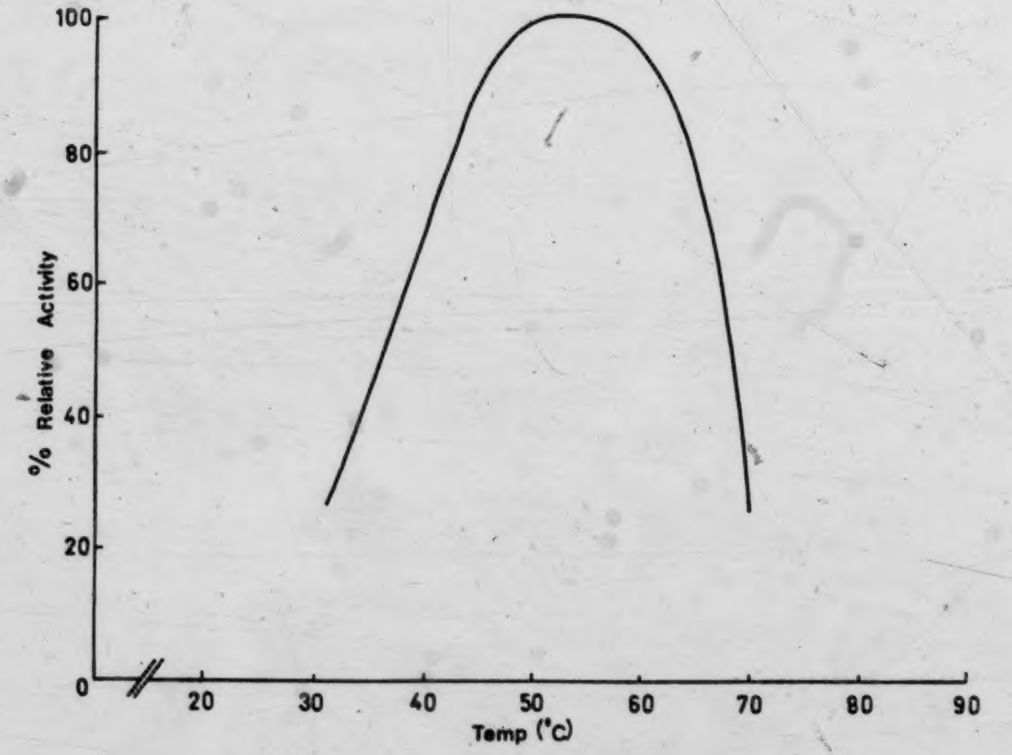

Figure 2.10. Effect of temperature on the activity of *Aspergillus oryzae* lactase(Anonymous, 1981d).

2.4.3 Assessment of enzyme performance.

The degree of hydrolysis, defined as the percentage of lactose molecules cleaved out of the total amount of molecules initially present, can be measured by the determination of the amount of lactose utilized or by the amount of glucose produced.

Lactose and its hydrolytic products glucose and galactose can be determined colorimetrically (Nickerson *et al.*, 1976), using High Performance Liquid Chromatography (HPLC) (Pirisino, 1981), with a cryoscope (Anonymous, undated, b) or by an enzymatic method developed and distributed by the Boehringer Mannheim Company which measures the reduction of nicotinamide adenine dinucleotide (NAD) when galactose is oxidized to galacturonic acid (Anonymous, 1980).

Lactose and glucose can be measured using a YSI sugar analyser model 27 (Yellow Springs Instruments Inc.). This instrument uses an immobilized oxidase enzyme coupled with a hydrogen peroxide sensitive electrode (Kosinski, 1981). Glucose in the sample reacts with the glucose oxidase in the presence of oxygen producing gluconic acid and hydrogen peroxide. The hydrogen peroxide is oxidized yielding a current proportional to the amount of hydrogen peroxide produced and hence to the quantity of glucose in the sample (Kosinski, 1981). This instrument is a very powerful tool for the determination of sugars in complex solutions. Only 25 µl of sample are required. No sample preparation is required for liquid samples, apart from dilution to an adequate linearity level. This minimizes a major source of error in most analytical techniques. The analysis can be performed much more rapidly than with the other methods; analysis time after dilution is approximately two minutes. The sugar content is displayed in less than one minute after injection of the sample, allowing repetitive tests in a short period of time. The reproducibility and precision of the method are excellent (Kosinski, 1981). No deep technical knowledge is needed for the performance of the analysis.

2.5 Lactose hydrolysis techniques.

Several processes for the conversion of lactose into glucose and galactose exist at a commercial, near commercial or significant pilot plant development stages. The hydrolysis of lactose may be catalysed either by the β-galactosidase enzyme or by acid (Coton, 1979). The methods can be classified as follows:

a.-Acid hydrolysis.
b.-Cation exchange resins.
c.-Free enzyme.
d.-Immobilized enzyme systems.

While the enzymatic hydrolysis may be suitable for whole whey, lactose solutions or ultrafiltration whey permeate, chemical catalysis processes are suitable only for hydrolysis of lactose in the absence of protein (MacBean, 1979).

2.5.1 Acid hydrolysis of lactose.

This process requires very high temperatures (usually about 140-150°C) and a very low pH (1.3-2.0), using mineral acids as catalysts (Macbean, 1979 ; de Boer and Robbertsen, 1981). Sulphuric acid has been the most commonly used catalyst. The reaction time ranges from 45 minutes to more than 24 hours, depending on the extremity of the conditions used. Very corrosion resistant heat exchangers are required, and the severity of the process gives a product with unacceptably high levels of off-flavour and colour (Macbean, 1979 ; Miller and Brand, 1980).

2.5.2 Ion exchange lactose hydrolysis.

Here the pH of the substrate (usually UF whey permeate) is brought to pH 1.2-2.0 by means of a strong acid cation exchange resin (Miller and Brand, 1980). The temperatures used are similar to the ones employed with mineral acids. Rapid exhaustion of the resin catalyst can be avoided by previous demineralization of the substrate. Microbial contamination is not a problem, because of the extreme conditions of hydrolysis. The brown colour developed can be removed using resins (Miller and Brand, 1980). No oligosaccharides are formed and since the process does not present any product inhibition problems, a 100% hydrolysis can be achieved. Economic considerations indicate that the ion exchange process could be competitive (Coton, 1979 ; de Boer and Robbertsen, 1981).

2.5.3 Free enzyme hydrolysis.

The simplest enzymatic method of lactose hydrolysis in whey is the direct addition of β-galactosidase to the whey in a batch process. This method has been successful for the lactose hydrolysis in milk (Dahlqvist *et al.*, 1977). The dosage of the enzyme is determined by the activity of the preparation used, the desired degree of hydrolysis, the temperature, pH and time of the reaction and the mineral content of the whey. The hydrolysis is terminated by inactivation of the enzyme by conventional HTST (High temperature short time) pasteurization. Most experience so far has been with the treatment of whole sweet whey using neutral enzymes (Burguess and Shaw, 1983). A temperature of 35-40°C and reaction times longer than 6 hours are recommended when using a neutral enzyme. Although some neutral β-galactosidase preparations are active at temperatures as low as -9°C (Baer and Loewenstein, 1979), this would not be relevant for the hydrolysis of whey. At low temperatures, the enzyme productivity is too low to be economical. The hydrolysis of concentrated whey at low temperatures is another possibility (Giec and Kosikowski, 1983). However, product inhibition could be a significant problem with a concentrated substrate.

Batch processes have many advantages: they are very easy to carry out and no additional or expensive equipment is required; with a free enzyme any type of lactose substrate can be used (none of the other hydrolysis techniques have given good results for the hydrolysis of whole whey), and the use of a soluble enzyme requires a low capital investment. The main disadvantage of this process is that after hydrolysis, the enzyme remains in the product and is therefore lost.

A process was developed to improve the utilization of the enzyme by recovering the enzyme using ultrafiltration techniques (Roger *et al*., 1976 ; Miller and Brand, 1980). This ultrafiltration process for enzyme recovery is unlikely to be used in the future because it is not economically attractive.

2.5.4 Immobilized enzyme systems.

A variety of methods have been used to immobilize β-galactosidases from different sources with varying degrees of success. Immobilization of enzymes involves the physical or chemical attachment to an insoluble matrix or within a confined volume (Finocchiaro *et al*., 1980). The most common methods of immobilization investigated intensively for possible industrial applications are listed in table 2.3.

Covalent binding of enzymes to insoluble supports provides a stable immobilized system, but preparation of these products can be complex and will require technical expertise. Expensive supports are often necessary, increasing the overall cost of the process. A substantial excess of enzyme is commonly used to obtain an acceptable amount of covalent attachment to the support. Methods to

Table 2.3. Methods of enzyme immobilization (from Finocchiaro *et al.*, 1980).

Chemical methods (covalent bond formation)	Physical methods (noncovalent bond formation)
Attachment to water insoluble matrix	Adsorption onto water insoluble matrix
Incorporation into growing polymer	Entrapment within water insoluble gel matrix
Intermolecular cross-linking with multifunctional, low molecular weight reagents	Entrapment within permanent and non-permanent semi-permeable micro-capsules
	Containment within semi-permeable membrane devices

maximize the utilization of the enzyme must be developed. The covalent attachment of *A. niger* lactase to functionalized controlled-pore glass beads is the most frequently used technique (Finocchiaro *et al.*, 1980).

Entrapment techniques, involving crosslinked polymers, microcapsules, fibers and membranes have also been used. Effective immobilization is dependent on a delicate balance of experimental factors, and this minimizes the use of these techniques in large-scale operations. Encapsulation is an attractive method for the immobilization of lactase. If both substrate and products permeate through the microcapsules an extremely large surface area to volume ratio can be achieved. However, the permeability of a substrate is solubility- and diffussion-dependent, constituting an important disadvantage (Finocchiaro *et al.*, 1980).

Cellulose fibers and membranous collagen have also been considered. The entrapment of enzymes in synthetic fibers is claimed to be well-suited for large scale operations. Cellulose acetate fibers are used in a successful commercial operation for hydrolysing lactose in milk. This process was developed in Italy by SNAM Progetti and uses a yeast derived β-galactosidase (Burguess and Shaw, 1983; Finocchiaro *et al.*, 1980). The Italian process seems to be the only immobilized enzyme application being used commercially with milk. It is carried out in a batch reactor at low temperatures and the final product is UHT(Ultra high temperature) processed for liquid consumption.

Among the physical methods, adsorption is probably the most simple and economical approach to lactose immobilization. A variety of materials have been used: alumina, stainless steel, glass, sand, anion exchange resins, cellulose, chitin, carbon, etc. Enzyme desorption after continuous use can be eliminated if the enzyme is crosslinked after its adsorption.

A number of immobilized enzyme systems have been developed for whey processing applications. A patented hydrolysis technology developed by Corning Glass is the best known system. The system utilizes an *A. niger* β-galactosidase immobilized on controlled pore silica beads, and has been reported to be in commercial use to produce lactose hydrolyzed syrups, whey protein concentrate and baker's yeast, in a joint venture of Corning and an american supermarket chain (Anonymous, 1981c). In England, the Milk Marketing Board and Corning have set up a joint company to produce and market a sweetener obtained from whey (Anonymous, 1982). The New Zealand Dairy Board is involved with Corning in a similar enterprise (Anonymous, 1982).

It is interesting to note a claim by Corning that a 20% total solids feed going into the continuous, downward flow, fixed bed reactor can be hydrolysed without any reduction in reaction rate or an increase in the formation of oligosaccharides. However, it is well known that an increase in substrate concentration leads to a reduction in reaction rate and a subsequent low degree of hydrolysis (MacBean, 1979).

A system developed by Rohm GmbH has been described recently (Sprosler and Plainer, 1983). An enzyme from *A. oryzae* is covalently bound in the pores of a macroporous, bead-shaped carrier. Studies on a large scale are necessary before the system can be considered attractive for the industry.

The hydrolysis of lactose in whey or whey permeate using immobilized enzymes involves a substantial capital investment and is appealing only for large scale operations. In the large majority of the existing applications, batch hydrolysis using a free soluble enzyme is utilized. Only a handful of immobilized systems are available for potential commercial operations. After the very considerable research effort that has been invested in immobilized β-galactosidase technology, the technique has not been as commercially successful as predicted. As was pointed out by Fox (1980), the reason may be that the problems to be solved may not be as commercially significant as many authors claim, and they can be solved by the simple use of the soluble enzyme or by other techniques.

2.6 Hydrolysis of lactose in acid whey.

A tentative process scheme for the hydrolysis of lactose in acid whey using a free soluble enzyme is given in figure 2.11. The pH of the whey is adjusted to the optimum pH of the enzyme being used. The enzyme is added and the

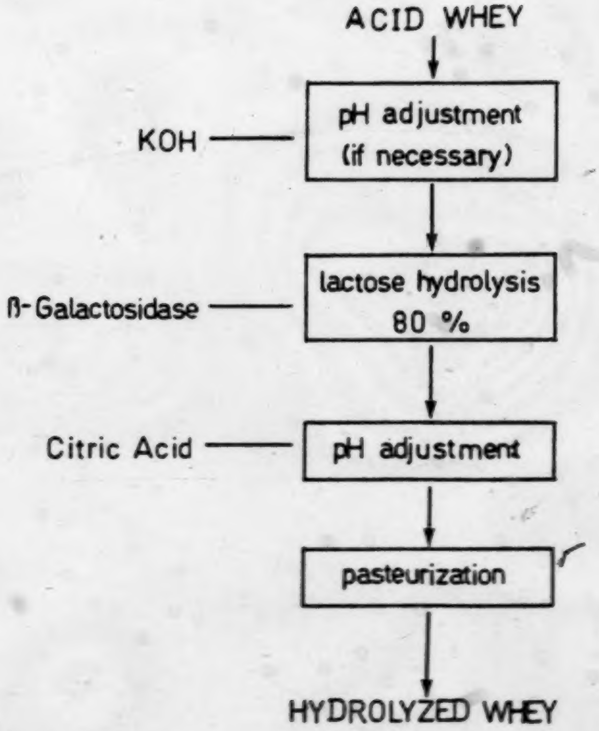
ACID WHEY
KOH
pH adjustment (if necessary)
ß-Galactosidase
lactose hydrolysis 80 %
Citric Acid
pH adjustment
pasteurization
HYDROLYZED WHEY

Figure 2.11. Process scheme for the hydrolysis of lactose in whey using a soluble β-galactosidase.

hydrolysis is carried out in a closed stirred tank at the optimum temperature of hydrolysis, again dependent on the enzyme. After a 75-85% hydrolysis is achieved, the whey is pasteurized. To minimize protein denaturation, pH readjustment should be done before the heat treatment. A demineralization step could be included after the hydrolysis and before the pH adjustment. Without being concentrated, the end product can be used as a beverage base. Since this base would contain hydrolysed lactose, whey proteins, calcium and other minerals, it could be used, after adequate flavouring, to produce a highly nutritive and refreshing thirst-quenching drink for sportsmen and lactose intolerant people.

2.7 Why use a free enzyme?

In theory, the concept of immobilized enzymes is highly attractive. The first developments in enzyme immobilization techniques created a lot of excitement. This excitement was further increased by the success of several industrial pilot plant operations. It was believed that immobilized enzyme technology was going to revolutionize the food industry. It may be surprising to find that only a few immobilized enzymes have been industrially successful. Only one process, the use of glucose isomerase for the conversion of glucose into fructose in the production of high fructose corn syrup, has been successful on a large commercial scale (Hultin, 1983). It has been recognized, with some disappointment, that immobilized enzymes are not "the wave of the future". Certain factors are responsible for the very critical approach that has been seen on the industry side. These factors represent the pragmatic view of commercial investment and operating practice (Godfrey, 1983). Even after many laboratory reports showing good possibilities for a process, the economics may not justify attempts to scale up the idea in the face of low cost soluble enzyme technology. A substantial increase in the use of soluble enzymes has occurred (Jelen, personal communication), while the introduction of immobilized lactases has been very slow (Godfrey, 1983). One of the main limitations to the use of immobilized enzymes is their stability after immobilization. If significant stabilization is not achieved, the procedure cannot compete well with the use of soluble enzymes (Hultin, 1983). Soluble enzymes are capable of transforming a much greater amount of substrate than normally achieved by the same enzyme after immobilization (Fox and Morrisey, 1980), and have obvious kinetic advantages (Miller and Brand, 1980).

Industrial interest in immobilized lactases will exist if some special properties of the product can be obtained only with the use of immobilized systems. This is not the case for the hydrolysis of lactose in whey, since a product of the same or better quality can be obtained with the soluble enzymes. Another reasons for the slow response from the industry is related to the use of whole whey as a substrate. Problems associated with protein adsorption to a variety of enzyme support systems and the mainteinance of acceptable column sanitation levels limit the usefulness of an immobilized lactase.

The immobilized lactase system has its best chance with the hydrolysis of large volumes of ultrafiltration whey permeate (Coton, 1979). The removal of protein from the whey makes it necessary to include an additional and expensive processing step, the ultrafiltration. In the development of any new technology on a commercial level there has to be an economic advantage for the adoption of the new technology to occur. For the immobilized lactase system to be economically feasible, volumes of 200,000 litres of permeate a day per plant are required (Dohan *et al.*, 1980). This is sometimes found in Europe but rarely in Canada. In the province of Alberta, typical volumes of acid whey produced are in the order of 20,000 litres daily per plant. The simplest method of achieving lactose hydrolysis in this conditions is the direct addition of the enzyme into the whey.

In light of the factors mentioned previously, the use of a free soluble β-galactosidase appears to be the most appropriate choice for the hydrolysis of lactose in cottage cheese whey in Alberta.

2.8 Physicochemical properties of the proteins.

The physicochemical properties of the whey proteins are relevant to the first part of this study, where the thermal stability of whey systems and isolated whey proteins were investigated.

Whey proteins or milk serum proteins are the nitrogen compounds remaining in the milk serum after precipitation of the caseins at pH 4.6. Nearly one fifth of these non casein nitrogen compounds are not real proteins, consisting of non-protein nitrogen and proteose-peptones (de Wit, 1981).

Most of the milk proteins are heterogeneous. Genetic variants of β-lactoglobulin were the first to be recognized; soon after that the variants of the other proteins were also discovered. According to Swaisgood (1982), there are two known genetic variants of α-lactalbumin, six of β-lactoglobulin and eighteen of the various caseins altogether.

The caseins and the serum proteins show substantial qualitative differences. The caseins are associated into micelles, are not soluble over the whole pH range and exist in a "naturally denatured state" (Dalgleish, 1981). In contrast, the whey proteins are globular proteins susceptible to heat denaturation (McKenzie, 1970; Dalgleish, 1981). The structural differences are due to the presence of numerous disulphide bonds in the whey proteins, while the caseins possess few cysteine residues, and are unable to stabilize their structures by disulphide bridge formation.

Another important difference is that the caseins are phosphorylated, a property that determines their extensive capacity to bind divalent metal ions and undergo complex precipitation reactions (Dalgleish, 1981).

The major protein constituents of whey are β-lactoglobulin, α-lactalbumin, bovine serum albumin and the immunoglobulins. They differ both in structure and in properties as a result of differences in amino acid sequence (de Wit, 1981). While β-lactoglobulin and α-lactalbumin are synthesized in the mammary gland, bovine serum albumin and the immunoglobulins are derived from the blood (de Wit, 1981).

Some of the properties of these proteins are summarized in table 2.4. The sulphur contents of the major whey proteins are; β-lactoblobulin, 1.6%; α-lactalbumin, 1.9%; and bovine serum albumin, 1.9%. The immunoglobulins comprise a mixture of glycoproteins of different sizes and are grouped together because they have a common antibody activity (Marshall, 1982). These immunoglobulins consist of numerous different kinds of protein molecules and because of this heterogeneity they were not included in the present study.

During heat treatment, the unfolding of the globular structure of the whey proteins produces a more random coil conformation that is more susceptible to protein-protein interactions, resulting in self-aggregation via Ca^{2+} bridges, disulphide interchange or hydrophobic bonds (Morr, 1982). In particular, β-lactoglobulin and BSA govern protein

Table 2.4.- Some properties of the major whey proteins; adapted from de Wit (1981) and Marshall (1982).

Whey protein	Molecular weight (daltons)	Conc. in whey (g/l)	% of total whey protein	Isoelectric point (pH)
β-lactoglobulin	18400	3.0	50	5.2
α-lactalbumin	14200	0.7	12	5.1
Immunoglobulins	160000	0.5	8	5.5-6.8
Serum Albumin	69000	0.3	5	4.8

aggregation through initiation of thiol/disulphide interchanges and oxidation/reduction reactions, although α-lactalbumin participates in these heat induced reactions to a lesser degree (de Wit, 1981).

The thermal behaviour of the whey proteins is affected by environmental conditions such as pH, protein concentration and the presence of salts and sugars. The effects of these factors are interdependent.

The pH regions of particular practical interest are; the isoelectric region near pH 4.0 to 6.0 where these proteins are most heat sensitive; the region between pH 6.0 and 7.0, where the heat stability of milk seems to be closely related to the thermal behaviour of β-lactoglobulin; and the pH region 2.5-3.5, where these proteins retain their good solubility (de Wit, 1981).

Since the number of particles is a very important factor in the kinetics of the thermally induced protein-protein interactions, an increase in the concentration of proteins present will affect their thermal behaviour (de Wit, 1981).

It is known that sugars and polyhydric alcohols stabilize globular proteins against thermal unfolding, and that some Maillard reactions occur during milk processing. The role played by the sugars in the heat stability of the protein is very pH dependent (de Wit, 1981).

The presence of calcium ions influences the extent of whey protein aggregation. The sensitivity to calcium flocculation is increased above pH 6.5, probably due to an increase in thiol reactivity (de Wit, 1981).

2.8.1 Bovine β-lactoglobulin.

In physiological conditions this protein exists as a dimer with a molecular weight of 36,700 daltons (Swaisgood, 1982). An important feature of the primary structure of β-lactoglobulin is the presence of two disulphide bonds and a thiol group, giving the molecule an opportunity for intra or intermolecular thiol disulphide interchange. The secondary structure is 10-17% α-helix, 24-42% antiparallel β-structure and the rest as unordered structure (Sienkiewicz, 1981a).

Between pH 3.5 and 5.2, the dimers associate to form octamers of roughly 147,000 daltons. This octamerization is the highest at pH 4.4-4.7, near 0°C (Sienkiewicz, 1981a). Below pH 3.5, the dimer reversibly dissociates due to strong electrostatic repulsive forces which develop as the ionogenic groups on the surface of the molecule are titrated. In the alkaline region, some conformational changes occur and an increase in thiol activity is observed.

2.8.2 Bovine α-lactalbumin.

This protein participates in the synthesis of lactose as the noncatalytic subunit (component B) complexed with galactosyl-transferase (Shukla, 1973), and has recently been shown by Hiraoka *et al.*(1980) to be a calcium metalloprotein. It undergoes an acid transition below pH 4.0 due to the loss of a tightly bound Ca^{2+} (Swaisgood, 1982).

At pH 6.8-7.0, the proposed secondary structure includes 26% α-helix, 14% β-structure and 60% unordered structure (Sienkiewicz, 1981b). Four disulphide bonds are present in the molecule.

This protein is highly homologous to hen's egg white lysozyme in primary structure and it is believed that their three-dimensional structures are also similar (Swaisgood, 1982). However, α-lactalbumin is less stable than lysozyme and the thermodynamic parameters of unfolding are different for the two proteins (Pfeil, 1981). The structure of α-lactalbumin in solution seems to be more flexible than the lysozyme structure.

2.8.3 Bovine serum albumin.

This has been one of the most studied proteins for many years, and it accounts for 60% of total protein in the blood plasma. It is synthezised in the liver and transported into milk through the mammary cells or by "leaky junctions" between the secretory cells in some cases of mastitis (Jenness, 1982).

Serum albumin is a single chain protein cross-linked by seventeen disulphide bridges, and with an α-helix content of 68%. A very interesting property is its ability to bind fatty acids and other ligands through hydrophobic interactions. This fatty acid transport seems to be BSA's most important function and is a means of transporting sparingly soluble substances between tissues or organs (Brown and Shockley, 1982).

2.9 Protein denaturation and aggregation.

2.9.1 Definition of protein denaturation.

Protein denaturation is defined as a major change from the original native state of a protein without alteration of its primary structure (Tanford, 1968). The term unfolding is often used in place of denaturation.

The folded, well-defined, essentially rigid three-dimensional structure in which most proteins exist in living organisms is known as the native state of a protein (Tanford, 1968). This compact, globular conformation reflects the existing physiological conditions and is essential for the biological function of the protein (Pace, 1975; Lapanje, 1978).

The native structure of a protein includes all four primary, secondary, tertiary and quaternary structures. Primary structure refers to the amino acid sequence of the protein. Secondary structure denotes the hydrogen bonds involved in α-helix or β-sheet structures. Tertiary structure describes the pattern of folding into a compact globular structure. Finally, the quaternary structure refers to non-covalent association of two or more polypeptide subunits (Lapanje, 1978).

The definition of denaturation involves cooperative changes of the secondary and tertiary structures in a single-chain protein. These changes are called cooperative because many amino acids are involved in a steep transition over a narrow range of temperature or denaturant concentration (Tanford, 1968). A change in quaternary structure is considered as denaturation only if it is accompanied by a major conformational change. The term denaturation does not apply to the changes undergone by proteins which have little or no ordered structure in their native states.

Protein denaturation, being a physicochemical process, can be reversible or irreversible. If the native structure and some other characteristic properties are regained after restoring the original conditions, the denaturation is considered to be reversible (Lapanje, 1978). Denaturation can be brought about in many ways. The most frequently used protein denaturants are pH, heat, urea and guanidine hydrochloride (GdnHCl).

The tendency for unfolding and denaturation is favoured at pH values away from the isoelectric point of a protein, because of electrostatic interactions between like charges (Lapanje, 1978). Urea and GdnHCl are widely used because no other denaturant gives a greater extent of unfolding (Pace, 1975). Proteins in 6M GdnHCl with their disulphide bonds broken show a configuration very close to a random coil. With urea, higher concentrations are required to complete the unfolding.

Thermal denaturation is brought about by heating. This oldest and the most widely used treatment during food processing and preparation, was the first known type of protein denaturant (Tanford, 1968; Lapanje, 1978).

2.9.2 Measurement of protein denaturation.

Before the advent of techniques for characterizing the state of protein molecules in solution, the loss of solubility was the principal physical criterion used for determining the extent of denaturation. Protein aggregation and precipitation were considered synonyms of protein denaturation (Tanford 1968).

The unfolding of the native protein after heat treatment is usually followed by aggregation, so that denaturation often manifests itself as loss of protein solubility. However, protein unfolding and protein aggregation are two different processes (de Wit, 1981).

Aggregation is a collective term for protein-protein interactions (Hermansson, 1979). The usually reversible changes on the molecular level characterized by weak bonds at specific binding sites, are refered to as protein association (e.g. monomer-dimer reactions). This should not be confused with aggregation. The terms aggregation, coagulation and flocculation involve unspecified protein-protein interactions and the formation of complexes with higher molecular weight (Hermansson, 1978).

Flocculation is a colloidal phenomenon determined by the balance between electrostatic repulsion and Van der Walls attraction; the tendency for a protein to flocculate is high in its isoelectric pH region. Coagulation is the random aggregation which includes denaturation of protein molecules; when protein-solvent rather than protein-protein interactions are favoured, no aggregation or coagulation are likely to occur (Hermansson, 1979)

Many methods and techniques are used to study protein denaturation. Included among them are optical methods such as Optical Rotatory Dispersion (ORD), Circular Dichroism (CD), ultraviolet (UV) and infrared (IR) absorptions, Fluorescence Spectroscopy and Light Scattering, and methods based on transport processes, like viscosity, sedimentation and diffusion. Calorimetry is another very useful method for the study of protein denaturation (Lapanje, 1978).

The use of calorimetry is still restricted to a relatively small number of laboratories, although this situation is expected to change in the next decade. It has been said (Edsall and Gutfreund, 1983) that there are as many spectrophotometers in a single major university as there are calorimeters in all the biochemical laboratories in the world.

Calorimetric studies distinguish between the denaturation and aggregation processes involved in the formation of protein precipitates. Turbidity measurements may be used to follow aggregation reactions, but they have the disadvantage of not being able to tell whether an increase in turbidity is due to changes in number, in size or in the optical properties of the particles (Hermansson, 1979).

While the unfolding of the protein molecule can be studied by calorimetry, the extent of precipitation can be determined from the protein content of the supernatants following centrifugation, before and after heat treatment. Several methods are available for protein determination; probably the more commonly used are the total nitrogen determination by Kjeldahl and the protein determinations by the Biuret and the Folin-Lowry methods.

The Folin-Lowry method was considered most adequate for the present work. In contrast with the Biuret method, the sensitivity is high and the presence of lipids and carbohydrates does not interfere with the Folin-Lowry determination (McKenzie, 1970). The method is based on the reaction of the tyrosyl groups of the proteins with the chromogenic reagent of Folin and Ciocalteu. Considerable attention must be given to the calibration for every protein and for every modification of the method (Lowry *et al.*, 1951).

2.10 Differential Scanning Calorimetry.

Differential Scanning Calorimetry (DSC) is an instrumental calorimetric technique used to determine the rate and energy involved in the physicochemical changes occurring in a system as a function of temperature.

There is an absorption or liberation of heat as a system or a material undergoes a change in state. This change can be the result of a melting process, a crystalline transition or a chemical reaction (Wright, 1982). The technique is called DSC because it measures and compares the rate of heat flow to a sample and to an inert reference which are heated or cooled at the same relative speed.

If the sample and the inert reference are maintained at the same temperature there will be a differential heat flow when the sample undergoes any thermally induced changes. These heat-associated changes are recorded on a thermogram (Figure 2.12). The direction of the peak indicates whether the heat flow is exothermic or endothermic. When the differential heat flow is recorded against time, the area under the curve is proportional to the heat change associated with the transition. The temperature of the transition can be determined from a thermogram of the differential heat flow against temperature.

The recording of the differential heat flow and the transition temperature are done independently for calorimetric accuracy. The sample temperature is stationary during the transition, and if the enthalpy of this transition were recorded against the temperature, the area under the curve would be distorted.

DSC had not been a very commonly used technique until the recent developments in the sensitivity of commercially

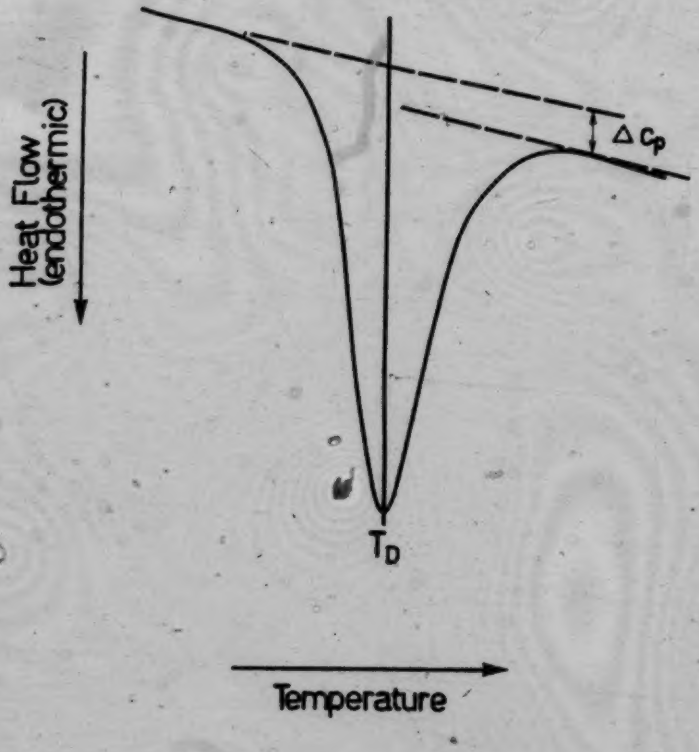
Heat Flow (endothermic)
Δ Cp
T_D
Temperature

Figure 2.12. Protein denaturation thermogram obtained by Differential Scanning Calorimetry.

available instruments (Wright, 1982; Biliaderis, 1983). Most of the modern equipment operate with a sample size of 10-20 mg (Lund, 1983). Following the development of the instrumentation in the last decade, DSC is now a widely used tool in thermal study of foods and their components.

2.10.1 Applications of DSC in food protein systems.

DSC has been used extensively to study protein denaturation. Other important phase transitions in foods that can be monitored by this technique are water freezing and starch gelatinization (Lund, 1983). Basic information on the denaturation of food proteins during processing can be obtained under conditions similar to the ones of the actual food process.

In proteins, DSC detects the thermally induced structural melting or unfolding of the molecule resulting in protein denaturation. Fundamental denaturation studies with isolated proteins and more practical studies of different aspects of protein behaviour related to food quality and processing, have been the main applications of DSC in food protein research (Wright, 1982). Some of the proteins used in these studies have been soy proteins (trypsin inhibitors, 7S and 11S globulins), egg white proteins (lysozyme, ovalbumin, avidin), meat or muscle proteins (collagen, myosin, myoglobin), and milk proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin).

2.10.2 DSC studies of whey proteins.

Casein, the major protein component of milk, already exists in a random coil configuration in the native state and consequently does not exhibit any denaturation when observed by DSC. In contrast, the whey proteins are heat sensitive, a characteristic that affects their structure and physicochemical properties (de Wit, 1981).

When DSC was used to examine the thermal transitions of whey proteins by Ruegg *et al.* (1977), the position of the denaturation peaks was affected by the heating rate. This indicates that maximum deflection temperatures cannot be quoted without reference to heating rate and that the denaturation temperatures should be extrapolated to a zero heating rate. Conformational changes starting just above 50°C were observed for α-lactalbumin, which until then had been regarded as the most heat resistant whey protein (Itoh *et al.*, 1976). Furthermore, the thermal denaturation of this protein was shown to be reversible upon cooling, another useful type of information easily obtained with DSC.

Gumpen *et al.* (1979) demonstrated the advantages of DSC in ligand-bovine serum albumin binding studies. The protein molecule was stabilized in a similar manner by binding of lauric or stearic acids or sodium dodecyl sulphate, although the best degree of stabilization was given by lauric acid.

The whey protein most often studied by DSC appears to be β-lactoglobulin. The role of water on the denaturation of this protein was investigated by Ruegg et al (1975). The changes in the thermal stability of the protein molecule showed a strong correlation with the degree of hydration.

The pH-dependence of the thermal stability of β-lactoglobulin in the pH range 6.4-7.3 was described by Ruegg *et al.* (1977). Hegg (1980) confirmed this pH dependence in a wider pH region (2.0-9.0), finding a maximum stability in the pH range 3.0 to 4.0 which decreased gradually as the pH was shifted toward the alkaline region.

A kinetic study of the denaturation of β-lactoglobulin at pH 6.7 was described by de Wit and Swinkels (1980). They found that up to the peak temperature, the denaturation followed first order kinetics, but above this temperature the behaviour was affected by secondary processes such as irreversible protein aggregation. Since DSC is an indirect analytical technique in the sense that it cannot usually define the precise nature of a particular molecular process (Wright, 1982), additional physicochemical data from the literature obtained by other analytical techniques were necessary to support this conclusion.

Harwalkar (1980) measured the thermal denaturation of β-lactoglobulin at pH 2.5. No aggregation was observed after the molecules had been unfolded. Several techniques rather than DSC alone were applied to examine the differences between the denaturation process occurring in these conditions and that taking place at neutral pH.

The thermal reactivity of β-lactoglobulin in temperatures up to 160°C was studied by de Wit and

Klarenbeek (1981). The high thermal sensitivity of this protein at the normal pH of milk and the effect of this sensitivity on the other milk constituents, were suggested to play an important role in the changes arising during high and ultrahigh heat treatment of milk and milk products.

The protective effect of glucose and lactose on the thermal behaviour of the protein was also investigated in their interesting study. The modification of the water structure of the protein molecule by the sugars was the explanation given for the increase in the denaturation temperatures observed.

Varunsatian *et al*. (1983) used DSC, in addition to other techniques, to evaluate the influence of Ca, Mg and Na on the heat aggregation of whey protein concentrates (WPC). In comparison to WPC without any salt, the denaturation temperatures were slightly higher with NaCl, and 2-4°C lower in the presence of Ca or Mg.

Another work with WPC was recently published by de Wit *et al*. (1983). The extent of protein denaturation in several WPC's and in the individual isolated whey proteins (α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulins) was analyzed by DSC. These researchers found significant differences (between 35 and 91%) in the amounts of undenatured protein present in several commercial WPC's analyzed, presumably caused by the different protein separation techniques used during their preparation.

The heat stability of most of the protein-containing whey products will be determined primarily by the physicochemical properties of the whey proteins. In the case of a whey-based beverage, this heat stability will be critical for the final quality of the product. The formation of a protein sediment after storage is an unpleasant characteristic that is often noticeable in whey beverage products (Jelen and Bucheim, 1984) and that may influence their acceptability.

Perhaps the easiest way to solve this problem would be to remove the heat-sensitive protein before the heat treatment is applied. However, the excellent nutritional value of all the whey proteins makes it imperative that they be retained in products marketed as nutritious beverages containing valuable dairy components.

3. MATERIALS AND METHODS.

3.1 Introduction.

The experimental work was divided into three major parts: 1) the whey protein heat denaturation and precipitation studies; 2) the lactose hydrolysis experiments carried out for the comparison of the commercial β-galactosidase preparations; and 3) the sensory evaluation of prototype drinks prepared according to the results obtained in the first two parts. The analytical procedures used in all three parts are described at the end of this chapter.

3.2 Protein denaturation and precipitation.

This investigation consisted of two sections, the study of the effect of pH on the heat precipitation of the whey proteins and the protein denaturation experiments carried out using Differential Scanning Calorimetry.

3.2.1 Protein precipitation experiments.

For the study of the heat stability of whey protein, acid whey was prepared by isoelectric precipitation (pH 4.65) of previously skimmed raw milk obtained from the University of Alberta Dairy Farm. The casein was removed by centrifugation at 27000 x g for 30 minutes (Beckman Model J2-21 Centrifuge; Beckman Instruments, Palo Alto, California).

The pH of the acid whey samples was adjusted with 2.0 N HCl. After pH adjustment, the samples were centrifuged (5000 x g for 20 minutes) to remove any possible turbidity. The protein contents of the supernatants were determined by the Folin-Lowry method (Lowry *et al*, 1951). The samples were then placed in test tubes which were closed and heated in a water bath at 95(±0.5)°C for 5 minutes. The final desired temperature in the test tubes was reached in less than two minutes. Upon completion of the heating, the samples were cooled to 20-25°C in an ice bath, and the precipitates were removed by centrifugation (3000 x g for 20 minutes). The protein content of the supernatants was determined as described above, and the extent of protein precipitation was calculated as:

$$\% \text{ precipitation} = \frac{\text{protein in supernatant after heating}}{\text{protein in supernatant before heating}} \times 100$$

Two different batches of whey from two different milk lots were used for this study. Each experiment was carried out in triplicate, because preliminar experiments did not show great variations among the several repeats.

3.2.2 Differential Scanning Calorimetry experiments.

Whey protein concentrate (WPC) used in the heat denaturation studies was produced in our pilot plant by ultrafiltration of acid whey at room temperature in a DDS laboratory ultrafiltration module model Lab-20 (De Danske Sukkerfabrikker, Denmark), using GR6P membranes (25000 MW cut off).

Samples of α-lactalbumin (α-la) (No.L-4379), β-lactoglobulin (β-lg) (No.L-0130) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis Mo., U.S.A.) and used without further purification. Two BSA preparations were used; a standard product containing 1.0-1.3 mol of fatty acids per mol of albumin (No.A-7638), and an "essentially fatty acid free" preparation with less than 0.005% fatty acids (No.A-7511), according to the manufacturer (Sigma, 1982). Each of these proteins was suspended separatedly in a salt solution known as simulated milk ultrafiltrate (SMUF) (Jenness and Koops,1962). The protein concentration of these solutions was 100 g/l.

The DSC thermograms were recorded in a DuPont thermal analyzer model 990 (Du Pont Company; Wilmington, DE). Samples of the protein solutions (20 μl) were sealed into hermetic aluminum pans and heated from 10 to 130°C in the DSC cell. A pan containing 20 μl of distilled water was used as a reference. The heating rates were 2, 5, 10 and 20°C/min. Each experiment was carried out in triplicate. The temperatures of denaturation were estimated by extrapolation of the temperatures of maximum deflection obtained at different heating rates to a heating rate of 0°C/min.

The effect of pH on the denaturation temperature of WPC and isolated α-la, β-lg and BSA in SMUF were studied over the pH range of 2.5-6.5. The effect of milk sugars on the thermal stability of β-lg over the same pH range was studied by the addition of either 14.6 mM lactose or 14.6 mM glucose and 14.6 mM galactose to a β-lg solution. These sugars were obtained from Fisher Scientific Company (Fair Lawn, New Jersey). To estimate the renaturation of the different proteins, previously denatured samples were cooled down to 10°C in the calorimeter cell at a rate of 10°C/min and then rescanned. To study the importance of calcium binding on the thermal stability of α-la, 0.1 M EDTA was added to a portion of this protein solution. The pH adjustment of the samples was the last step performed prior to the recording of the DSC thermograms. All chemicals were reagent grade quality or better.

3.3 Lactose hydrolysis experiments.

3.3.1 Enzymes.

Six commercial lactase preparations were obtained from different enzyme suppliers. In the text, these enzymes will be referred to as follows.

A - MAXILACT L2000 from GB Fermentation Industries (2055 Rue Bishop Street, Montreal, Quebec, H3G 2E8), a liquid preparation from *Kluyveromyces lactis*.

B - LACTOZYM 3000L HP from Novo Enzymes (Van Waters & Rogers, 2700 Rue J.B. Deschamps, Lachine, Quebec, H8T 1E1), a liquid preparation from *Kluyveromyces fragilis*.

C - TAKAMINE BRAND FUNGAL LACTASE 30,000 from Miles Laboratories (P.O. Box 932, Elkhart, Indiana, USA, 46515), a powdered preparation from *Aspergillus oryzae*.

D - ENZECO FUNGAL LACTASE from Enzyme Development Corporation (2 Penn Plaza, New York, N.Y., USA, 10121), a powdered preparation from *Aspergillus oryzae*.

E - LACTASE PREPARATION 2214C from Rohm GmbH (Scott Laboratories Ltd., 950 Brock Road South, Pickering, Ontario, L1W 2A1), a powdered preparation from *Aspergillus oryzae*.

F - LACTASE-AIE from Amano International Enzyme Co., Inc. (P.O. Box 1000, Troy, Virginia, USA, 22974), a powdered preparation from *Aspergillus oryzae*.

Two of these preparations (enzymes A and B) were neutral lactases, while the other four were acid enzymes. All preparations were used without further purification.

3.3.2 Hydrolysis conditions.

The conditions of hydrolysis for the neutral enzymes were 38°C and pH 6.8. With the acid enzymes the hydrolysis experiments were performed at 55°C and pH 4.65. The pH adjustment, when necessary, was made with 5.0 M HCl or 5.0 M KOH. All the hydrolysis mixtures were stirred (1000 RPM) in 250 ml Erlenmeyer flasks throughout the reaction course in an Aquatherm Water Bath Shaker (New Brunswick Scientific Co. Inc., New Brunswick, N. J., USA)

For each of the enzymes, K_m and *V*max were determined at two different enzyme concentrations. Lactose solutions (2.9-29.0 mM in distilled water) were hydrolyzed for 10 seconds and the kinetic parameters were estimated from the initial velocity measurements using a computer program based on the statistical considerations of Wilkinson (1961).

The effect of potassium on the neutral enzymes was studied by obtaining the kinetic parameters in solutions of lactose (2.9-29.0 mM) in 0.025 M monopotassium phosphate buffer. Since the acid whey was neutralized with KOH prior to the hydrolysis, the K_m values obtained in the presence of potassium phosphate were the ones used for the neutral enzymes in the comparison.

In the preliminary experiments with *K. lactis* β-galactosidase, dispersions of bovine serum albumin (0.3 g/l), β-lactoglobulin (3.0 g/l), and ovalbumin (5.0 g/l) from Sigma Chemical Co. (St. Louis, MO) in the lactose solutions were used to test the effect of individual proteins on the rate of hydrolysis.

Acid whey for all the hydrolysis experiments was obtained from a local cottage cheese manufacturer and was used within two days upon arrival to our laboratory.

For all the enzymes, the hydrolysis of lactose in whey was determined as a function of time at several enzyme concentrations (0.1-3.0 g/l). The experimental data from the progress curves were fitted by nonlinear regression using the University of Alberta computer with a BMDP program (Ralston, 1979).

The effect of galactose on the acid enzymes was studied by adding different amounts of galactose (7.3-13.14 mM) to the whey prior to hydrolysis.

For the experimental progress curves obtained for each enzyme, six enzyme concentrations were used: 0.1, 0.3, 0.5, 1.0, 2.0 and 3.0 g of each enzyme preparation per litre of whey.

After completion of the specified time intervals of the respective hydrolysis experiments described, the enzyme was inactivated by placing the samples in boiling water for 3 minutes. All experiments were done in triplicate. The percentage of hydrolysis was defined as:

$$\%H = \frac{\text{mM glucose produced}}{\text{mM initial lactose}} \times 100$$

3.4 Sensory evaluation.

A grapefruit flavour (Givaudan Grapefruit Aroma 60750-74 from Givaudan Ltd., Dubendorf, Switzerland) was used in the preparation of several samples of a prototype drink. This prototype was prepared by adding 7 g of sugar to 100 g of cottage cheese whey (pH 4.6), followed by the addition of the flavour (0.01 ml). Citric acid (50% sol. in distilled water) was used to adjust the final pH to 3.65.

Three more drinks were made to determine if the use of some of the β-galactosidases had any negative effect on the sensory characteristics of the product. These drinks were prepared as the prototype described above, with the difference that the lactose in the whey was previously hydrolyzed to an 80% level using the enzymes considered to be the most adequate for this purpose (C, D and E) among the preparations evaluated. Sugar (4 g per 100 g of whey) was added to obtain a final sweetness similar to that of the original prototype drink. Triangle tests, performed following the procedures described by Larmond (1977), were used to detect possible differences in bitterness between the first product and the lactose-hydrolyzed samples. Six judges, previously selected for their capacity and consistency for detecting bitterness in quinone solutions, tested the samples in duplicate experiments which took place in two sessions to give a total of twelve judgments per test.

To compare the prototype product against a whey-based drink available on the market in Edmonton, a paired comparison test (Larmond, 1977) was carried out using 30 untrained panelists from the Department of Food Science of this University.

The evaluations took place in the Sensory Evaluation Room at the Department of Food Science of the University of Alberta. The individual tasting booths were illuminated with red light. The samples were identified by three digit random codes and served at 4-6°C after overnight refrigeration.

3.5 Analytical procedures.

3.5.1 pH.

The pH data for all samples used in this work were obtained with an Orion Model 601 A pH-meter (Orion Research Inc., Cambridge, Mass. USA).

3.5.2 Total solids.

Total solids of the whey and the whey protein concentrate produced by ultrafiltration were determined by overnight evaporation in a vaccum oven at 100°C (AOAC, 1980). The determinations were made in triplicate.

3.5.3 Protein.

Protein analyses of the whey and the ultrafiltration-prepared whey protein concentrate were carried out according to the Kjeldahl procedure (AOAC, 1980), using a conversion factor of 6.37% (from %nitrogen to crude protein).

The protein content of all other samples was determined using the Folin-Lowry method (Lowry *et al.*, 1951). Protein absorbance was recorded at 748 nm in a Beckman DU-8 Spectrophotometer (Beckman Instruments Inc., Irvine, California, USA). The protein used as reference was β-lactoglobulin from Sigma Chemical Co. (St. Louis, MO, USA).

3.5.4 Glucose and lactose.

The concentrations of lactose and glucose in all experimental samples were determined in a YSI Sugar Analyzer Model 27 (Yellow Springs, Ohio, U.S.A).

4. RESULTS AND DISCUSSION.

4.1 Acid stabilization of the whey proteins against heat-induced precipitation.

4.1.1 Introduction.

It is known that the heat treatment of whey protein products near neutral pH results in considerable losses in protein solubility (Hidalgo and Camper, 1977). At this pH, the heat denatured proteins interact via irreversible thiol-disulphide reactions to form protein aggregates which further group themselves into large sedimentable particles resulting in precipitation (Morr and Josephson, 1968). This heat-induced protein coagulation constitutes a serious problem in the manufacture of various whey products when the thermal processing is carried out under conditions which promote protein aggregation (Barlow *et al.*, 1984).

On the other hand, severe heating of whey at pH 2.5 produced no protein precipitation (Modler and Emmons, 1976). A reduced level of protein-protein interactions, due to the abscence of disulphide aggregation and to electrostatic intermolecular repulsion, seems to be the reason that whey protein precipitation at this pH does not occur (Harwalkar, 1979). The precise pH point at which whey proteins become resistant to thermal precipitation has not been well established.

The effects of heating whey acidified below pH 4.0 were recently investigated by Jelen and Bucheim (1984), using turbidity measurements. The stability of the whey system against visible change upon heating at 92°C for 15-30 minutes was attributed to a higher heat resistance of the whey protein at pH 3.7 or lower. However, turbidimetry is not able to differentiate between protein denaturation and protein aggregation; variations in number, size or optical properties of the particles in solution can produce changes in turbidity not necessarily related to protein denaturation.

The specific objectives of this part of the work were: 1) to determine the critical pH point of transition of the heat aggregation/precipitation stability of the whey proteins; 2) to use Differential Scanning Calorimetry (DSC) to determine the temperatures of denaturation of these proteins in the acid pH region; and 3) to study the influence of milk sugars and fatty acid content on the thermal behaviour of some of the individual whey proteins. The composition of the whey used in this study and the WPC prepared from it by UF are given in Table 4.1.

4.1.2 Influence of pH on the heat precipitation of whey proteins.

The effect of pH on the heat precipitation of whey proteins from acidified cottage cheese whey is shown in Figure 4.1. The transition from the soluble to the insoluble

Table 4.1.- Composition of whey and whey protein concentrate(WPC) prepared from it by ultrafiltration.

	Composition (g/100 g)		
	Protein	Lactose	Total solids
Whey	0.75	4.9	6.38
WPC	7.50	4.9	13.20

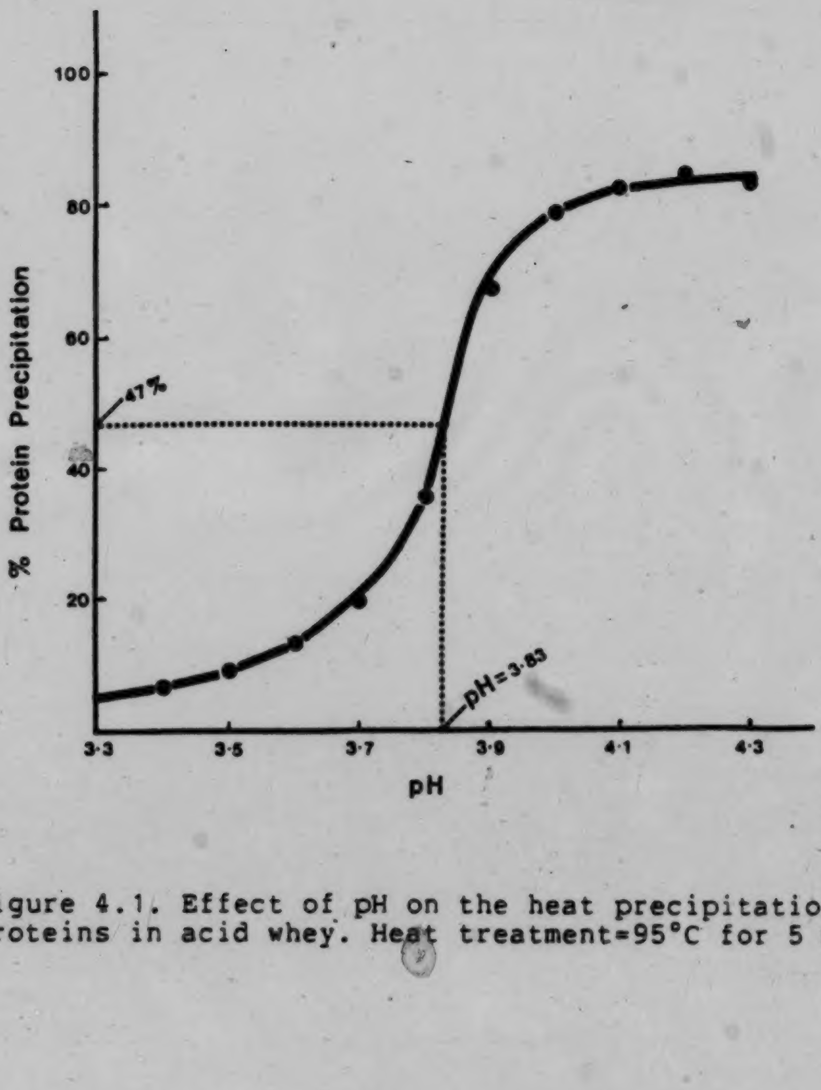

Figure 4.1. Effect of pH on the heat precipitation of whey proteins in acid whey. Heat treatment=95°C for 5 min.

or precipitated states ocurred within a narrow pH range (3.7-3.9). The protein contents of the supernatants before heating were not significantly different from the protein content of the original whey (Table 4.1). The differences in the amount of protein precipitated among the samples were therefore caused only by the heat treatment.

The low amount of protein precipitated below the critical pH region may be the result of high electrostatic repulsion between the denatured protein molecules, combined with the abscence of disulphide interchange reactions at such a low pH (Hermansson, 1979). Above pH 3.9, the repulsive forces are not so strong, protein-protein interactions are more likely to occur, and heavy protein precipitation is observed.

The pH dependence of the dissociation behaviour of β-lactoglobulin (β-lg) seems to support the previous argument. The β-lg dimer reversibly dissociates below pH 3.5 (Townend *et al.*, 1960; McKenzie, 1970). The monomerization of the native β-lg molecules is due to repulsive electrostatic forces developed as the pH is decreased (Swaisgood, 1982). The minor difference between the pH of transition to the monomeric state and the critical pH below which precipitation is minimal should not be interpreted so as to consider them totally unrelated phenomena. Bovine β-lg can dissociate to the monomer above pH 3.5; in fact, the dimer is still very weakly dissociated at pH 5.2. On the contrary, even at pH 2.7 the monomerization is not complete

(McKenzie, 1970; Mills and Creamer, 1975; Swaisgood, 1982).

A closer look into the heat denaturation of the whey proteins was necessary to establish whether the solubility of these proteins was retained because of the reasons discussed above, or due to the absence of protein denaturation when heating below pH 3.7.

4.1.3 Denaturation of whey proteins in the pH range 2.5-6.5.

The effect of pH on the denaturation temperatures of individual whey proteins and UF-prepared WPC is shown in Table 4.2. The thermal behaviour of the WPC seemed to be governed mainly by β-lg. The anomalous denaturation temperature observed for the WPC at pH 3.5 (88°C) could not be explained on the basis of the thermal behaviour of β-lactoglobulin alone. Perhaps a combined stabilizing effect exerted by the proteins or by the other constituents of the WPC and more perceptible at the conditions of total solids and pH at which this experiment was carried out could in some way be the reason for this unusual finding.

The results prove that, although they do not precipitate below pH 3.7, the whey proteins are still denatured by the heat treatment applied as in the precipitation experiment. The temperature range in which a protein is stable depends on the balance among the forces involved in the stabilization of its tertiary structure (Lehninger, 1973). Since some of these forces are pH dependent, it would be reasonable to expect variations in

Table 4.2. Effect of the pH on the denaturation temperature of α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA) and whey protein concentrate (WPC). The temperatures were extrapolated to a heating rate of 0°C/min.

pH	DENATURATION TEMPERATURES (±0.5°C)[1].			
	β-lg[2]	α-la[3]	BSA[4]	WPC[5]
6.5	75.9	61.0	71.9	76.9
5.5	77.8	61.2	72.6	78.8
4.5	81.2	61.5	74.0	82.1
3.5	81.9	58.6	73.5	88.0
2.5	78.7	–	–	80.6

---

[1] Average of three measurements at each heating rate (± average standard deviation from individual measurements).
[2] β-lactoglobulin.
[3] α-lactalbumin.
[4] Bovine serum albumin.
[5] Whey protein concentrate.

the denaturation temperature of a protein at different pH values, reflecting in some way the importance of these forces for the overall stability of the protein (Kinsella, 1981). In the case of β-lg, electrostatic interactions and perhaps other pH dependent forces seem to play a relatively important role in the stabilization of the three-dimensional structure of the molecule. In proteins like BSA, which are being exposed to a wide range of physiological conditions, noncovalent interactions are not the main forces responsible for the stabilization of the tertiary structure. Disulphide bonds, of which BSA has 17 (Brown and Shockley, 1982), may play a much more important role.

4.1.4 Influence of fatty acid content on the denaturation temperature of Bovine Serum Albumin.

The binding of polar compounds (i.e. fatty acids) to BSA may also be important in the stabilization of its structure, as is shown in Table 4.3. The stated difference between the two BSA preparations was the additional charcoal treatment to remove the fatty acids naturally bound to the protein, without altering its native structure. An increase in the thermal stability of a protein by ligand binding has been known for some time (Donovan and Ross, 1973; Donovan and Ross, 1975). A similar stabilization by fatty acid binding in BSA was observed by Gumpen *et al*. (1979) using DSC. The ability of BSA to bind hydrophobic ligands has very important physiological significance in the blood in the

Table 4.3. Effect of the fatty acids content in bovine serum albumin(BSA) on the maximum deflection temperatures of Differential Scanning Calorimetry thermograms at different heating rates. pH=4.5.

TEMPERATURE OF MAXIMUM DEFLECTION (±0.5°C)'.

Heating rate(°/min)	BSA	Defatted BSA
2	73.0	65.9
5	75.9	66.7
10	77.4	67.2
20	80.0	68.0

'Average of three measurements (± standard deviation) transport of such substances between organs and tissues, and in the neutralization of the toxic effects of some compounds by this binding (Brown and Shockley, 1982).

Table 4.3 also shows the effect of the heating rate on the maximum deflection temperatures obtained, and the importance of extrapolating to a 0°C/min heating rate when reporting the denaturation temperature of a protein.

4.1.5 Effects of milk sugars on the denaturation temperature of β-lactoglobulin.

The effects of lactose and a mixture of glucose and galactose on the thermal behaviour of β-lg are shown in Table 4.4. The denaturation temperatures over the pH range studied were higher when either lactose or glucose and galactose were added to the β-lg solution. The maximum deflection peaks obtained for the β-lg solution in the presence of glucose and galactose were higher than those obtained for this protein in the presence of lactose alone, in every single experiment carried out. Sugars and polyhydric alcohols are known to stabilize proteins against denaturation (Gerlsma, 1968; Gerlsma, 1970; Bechtle and Claydon, 1971). These substances tend to maintain or increase the hydration of the protein molecule, enhancing the water structure in its immediate surroundings, and contributing to its stability (Bull and Breese, 1978). The protein is preferentially hydrated in a sugar-containing aqueous system, where the macromolecule is stabilized

Table 4.4. Effect of milk sugars on the denaturation temperatures of β-lactoglobulin(β-lg) in simulated milk ultrafiltrate.

pH	DENATURATION TEMPERATURES(±0.5°C)[1].		
	A[2]	B[3]	C[4]
4.5	81.2	82.2	82.4
3.5	81.9	83.1	83.9
2.5	78.7	79.6	80.3

[1] Average of three measurements at each heating rate (±average standard deviation from individual measurements).

[2] Control, no sugar added.

[3] β-lg(100g/l) plus lactose (14.6mM).

[4] β-lg plus glucose(14.6mM) and galactose(14.6mM).

(Arakawa and Timasheff, 1982). Since this stabilization takes place at a molecular level, a slight further increase in the denaturation temperature was observed when the original sugar/protein molar ratio was doubled by the replacement of lactose by glucose and galactose.

4.1.6 Influence of calcium on the thermal behaviour of α-lactalbumin.

The binding of calcium by α-la seems to be essential for the stabilization of the tertiary structure of this protein. The drastic decrease in the thermal stability of α-la when calcium was removed by chelation with 0.1 M EDTA, shown in Table 4.5, provoked further study of the calcium stabilizing effect. The results of this study were accepted for publication (Bernal and Jelen, 1984a).

The enthalpy of denaturation and the reversibility of this denaturation observed for α-lactalbumin are presented in Table 4.6. Calcium binding seems to be fundamental also for the renaturation of this protein. None of the other proteins studied underwent renaturation. The high degree of renaturation of α-la may be caused by the recovery of the original structure of the protein by calcium binding upon cooling. The ability of α-la to regain its original structure disappeared when 0.1 M EDTA was added to the protein solutions. Using Circular Dichroism, Hiraoka *et al.* (1980) observed a qualitative difference in the stability of α-la during denaturation by guanidine chloride or heat in

Table 4.5. Effect of the removal of calcium by 0.1 M EDTA on the maximum deflection temperatures of Differential Scanning Calorimetry (DSC) thermograms of *α*-lactalbumin.

pH	TEMPERATURE OF MAXIMUM DEFLECTION (±0.5°C)[1].	
	α-la	*α*-la with 0.1 M EDTA
6.5	61.0	41.1
5.5	61.2	41.0
4.5	61.5	40.8
3.5	58.6	40.0
2.5[2]	–	–

---

[1] Extrapolated to a heating rate of 0°C/min (± average standard deviation from individual measurements).

[2] No thermogram peaks were obtained when heating in the 10-130°C range.

Table 4.6. Thermodynamic parameters of heat denaturation of α-lactalbumin(α-la) in simulated milk ultrafiltrate (SMUF) with and without 0.1 M EDTA.

pH	H(J/g)[1]		% Renaturation[2]	
	α-la	α-la/EDTA	α-la	α-la/EDTA
6.5	20.39±0.71	6.58±0.26	88	0
5.5	22.21±0.75	6.31±0.37	83	0
4.5	22.45±1.06	5.99±0.35	87	0
3.5	10.59±0.53	4.09±0.28	<50[3]	0
2.5[4]	--	--	--	-

---

[1] Enthalpy of denaturation (± standard deviation).

[2] Obtained from the ratio of the peak area of the first run over the peak area of the second run.

[3] Great variation (±50%) observed in replicate experiments.

[4] No thermogram peaks resulted from heating in the 10-130°C range.

the presence or abscence of EDTA. In the present work, the drastic decreases in the denaturation temperatures and enthalpies for α-la in the presence of EDTA at all pH levels studied confirmed the importance of the calcium binding for the heat stability of this protein.

It was not possible to obtain any thermogram peaks at pH 2.5. It is well known that α-la undergoes a conformational transition in the very acidic pH conditions (Swaisgood, 1982). The midpoint for this transition seems to be located around pH 3.3 (Contaxis and Bigelow, 1981). This acid transition could be the reason for the difficulties found when trying to record the thermograms at pH 2.5, and for the decrease in the temperature and the enthalpy of denaturation at pH 3.5.

The thermal denaturation of a protein may be reversible or irreversible, depending on the specific conditions under which the denaturation takes place (Ghelis and Yon, 1982). Due to the high calcium binding affinity, the native tertiary structure of α-la appears to be regained upon cooling as long as the calcium molecules are available to be bound again by the protein. This would not occur when EDTA or a high amount of hydrogen ions were present in the medium.

In the absence of EDTA, the denaturation of α-la was not nearly as reversible at pH 3.5 as at the other values. It was not possible to obtain reproducible thermograms during the rescanning of the samples; in all cases, the renaturation was well below 50%. If the acid transition of α-la was indeed due to the competition of the protons and the calcium ions for the same binding site, once that calcium has been removed as a result of the increased temperature, regaining the original position may have been difficult in this transition region. It appears that some calcium is still bound to α-la at pH 3.5, since at this pH the addition of 0.1 M EDTA produced a 18.6°C decrease in the denaturation temperature and more than 50% decrease in the denaturation enthalpy.

Some information on the nature of the calcium binding site in the α-lactalbumin molecule is now available in the literature. Kronman *et al*. (1981) found more than one cation binding site in the molecule and showed that zinc, manganese and cobalt were also bound by the protein, although with a lower affinity. Cation binding observed in several α-lactalbumin species (bovine, goat, human and guinea pig) suggested a sequence homology in the calcium binding site (Azuma *et al*., 1982; Murakami *et al*. 1982). Permyakov *et al*. (1982) proposed that the α-lactalbumin calcium binding region would be to some extent similar to the calcium binding site of parvalbumin and other calcium binding proteins.

Two physically distinct and mutually exclusive sites for calcium and zinc binding in α-lactalbumin were described by Murakami and Berliner (1983). It seems that since the protein cannot bind both cations simultaneously, the calcium form may be predominant under physiological conditions. The high similarity between the calcium binding site for α-lactalbumin and the nearly octahedral, oxygen coordinated binding site found in calmodulin, parvalbumin and troponin C (all calcium binding proteins) was demonstrated by Berliner *et al.* (1983). Further research into the mechanism of calcium binding in the transitory pH region is needed to elucidate the thermal behaviour of α-la and the reversibility of its heat denaturation process.

4.1.7 Remarks.

Like the changes in the quaternary structure of β-lg, the substantial differences in the amounts of total whey protein precipitated below and above pH 3.7-3.9 may be the result of the repulsion produced by the increase in the net charge on the surface of the protein molecules. Although, as shown by the DSC experiments, the heat treatment denatures the protein, the strong positive charges would prevent the unfolded molecules from coming together, form aggregates and then precipitate.

These results indicate that the problem of protein precipitation common to heat-treated whey-based beverages could be minimized by adjusting the pH of the drink to 3.6-3.7 before thermal treatment. The retention of the soluble proteins in the drink would bring obvious nutritional advantages to the final product.

4.2 Hydrolysis experiments using a *Kluyveromyces lactis* β-galactosidase.

4.2.1 Introduction.

While information about the kinetic characteristics of some lactases purified from commercial preparations can be found in the literature (Woychik and Wondolowski, 1972; Widmer and Leuba, 1979; Greenberg and Mahoney, 1981), only scarce data on commercial preparations without further purification are available. A substrate commonly used to define the activity of β-galactosidase is ortho-nitrophenyl β-D galactopyranoside (ONPG) (Richmond *et al.*, 1981). However, when an enzyme is applied to the actual substrates in the dairy industry, the only practical assessment is the determination of the β-galactosidase activity with the particular substrate, in this case, cottage cheese whey preparations.

In preparation for the main hydrolysis experiments, a yeast enzyme from *K. lactis* was used for the hydrolysis of lactose in neutralized acid whey. To identify some of the possible factors influencing the activity of this commercially available β-galactosidase and to determine the Michaelis-Menten constant (K_m) of the commercial enzyme preparation, several dairy substrates and model systems were also utilized. The enzymatic activity was tested in buffered lactose solutions, skim milk, cottage cheese whey, and whey permeate obtained by ultrafiltration of cottage cheese whey in a DDS laboratory ultrafiltration module (De Danske Sukkerfabrikker, Denmark). Lactose solutions (0.146 M) were prepared in 0.025 M potassium phosphate buffer, unless otherwise indicated. The results obtained in this preliminary hydrolysis study have been accepted for publication (Bernal and Jelen, 1984b).

4.2.2 Activity of *K. lactis* lactase in dairy substrates.

The practical objective of this study was to compare the lactase activity of the *K. lactis* neutral lactase preparation in skim milk, KOH-treated acid whey and KOH-treated acid whey UF permeate. The results are shown in Figure 4.2. The highest activity was observed in the whey, followed by the UF permeate and milk. Jakubowski *et al.* (1975) reported that the activity of soluble β-galactosidase from *Aspergillus niger* was similar in acid whey and in ultrafiltration whey permeate. Mahoney and Adamchuk (1980) observed that the activity for a β-galactosidase from *K. fragilis* was higher in skim milk than in whey and its permeate. These differences were likely due to the effects of the pH adjusting procedures used and possibly the source of the enzyme. In the work of Jakubowski *et al.* (1975), no neutralizing agent was used because the pH of the acid whey was adequate for the *A. niger* β-galactosidase. Mahoney and Adamchuk (1980) used sodium hydroxide to adjust the pH of their substrates.

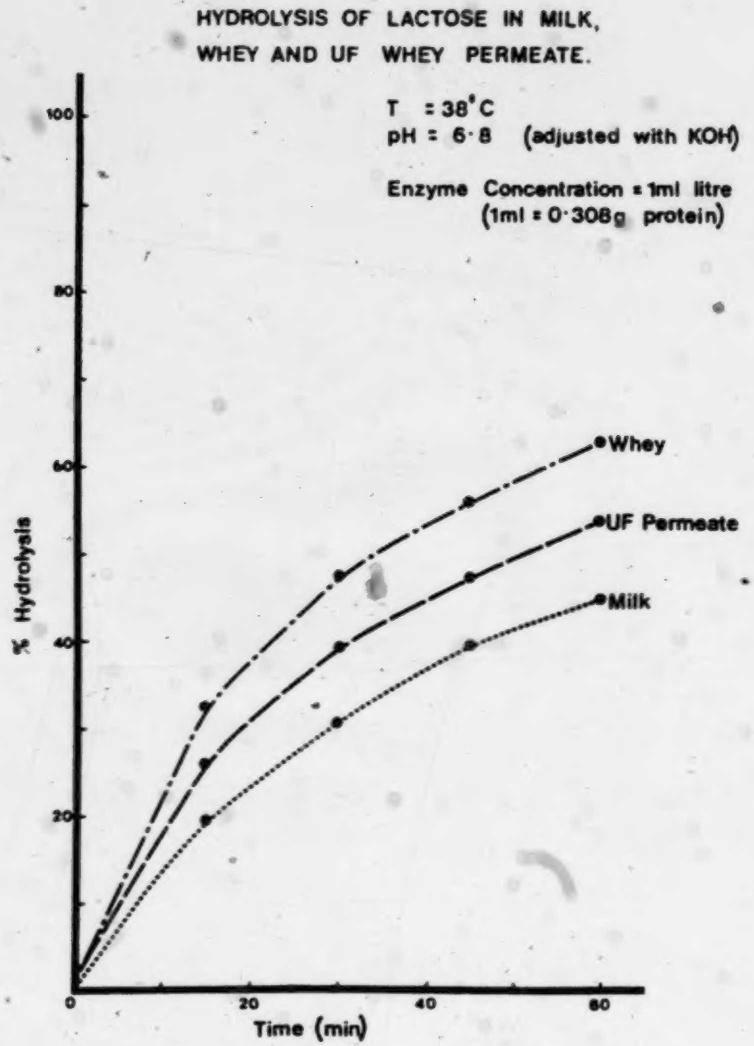

Figure 4.2. Hydrolysis of lactose in skim milk, whey and ultrafiltration whey permeate, using a *Kluyveromyces lactis* β-galactosidase preparation. Enzyme concentration=1 g/l.

Since potassium hydroxide is a much more suitable pH adjusting agent than sodium hydroxide (Figure 4.3), the former was used in our work to bring the pH of the acid whey and its permeate to pH 6.8, the optimum pH of the *K. lactis* enzyme. It is well known that while sodium inhibits the activity of this β-galactosidase, potassium increases it (Anon., undated, c; Burguess and Shaw, 1983).

4.2.3 Effect of whey proteins on the activity of *K. lactis* β-galactosidase.

The difference in the rate of hydrolysis between whey and UF permeate shown in Figure 4.3 prompted the investigation of the possibility that the whey proteins could have a similar activating effect on the *K. lactis* enzyme as suggested previously for the *A. niger* and *K. fragilis* enzymes (Jakubowski et al, 1975; Mahoney and Adamchuk, 1980). The hydrolysis of lactose in a model system was performed in the presence of β-lactoglobulin, bovine serum albumin, or ovalbumin. Ovalbumin was included to determine whether a non-milk protein could have a similar effect. No effect was observed with any of the three proteins added. The activity of *K. lactis* β-galactosidase in the protein-containing lactose solutions was similar ($P=0.02$) to the activity in the control (Table 4.7). This is in contrast to the results of Mahoney and Adamchuk (1980), who reported 2.5, 4.3 and 5.1 fold increases in the relative activity of β-galactosidase from *K. fragilis* in the presence

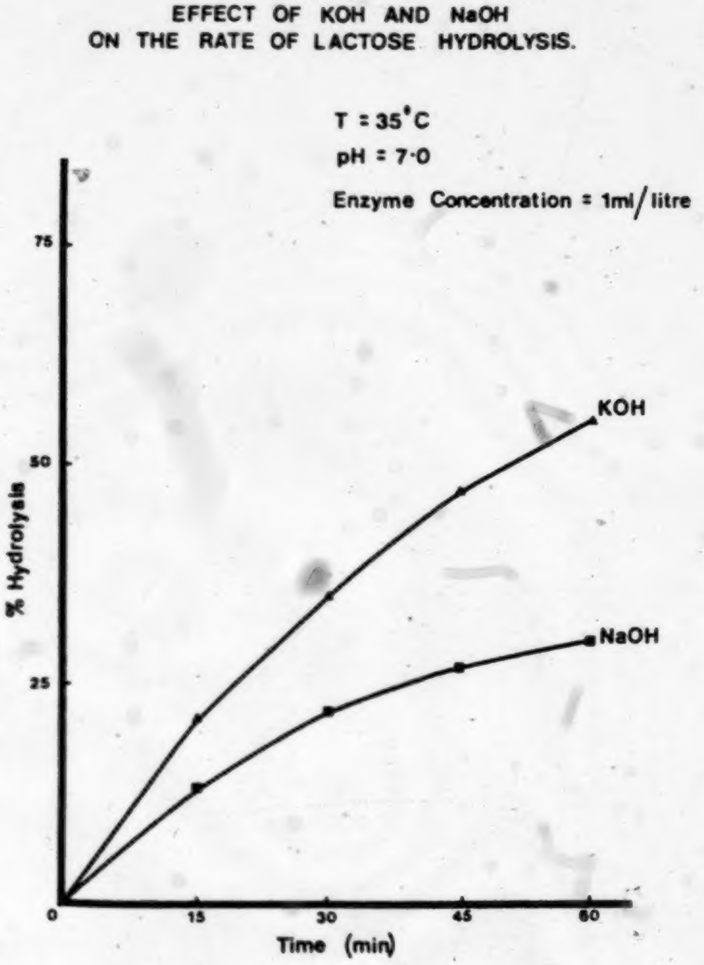

Figure 4.3. Effect of potassium (5.0 M KOH) and sodium (5.0 M NaOH) on the rate of hydrolysis in neutralized acid whey. Enzyme concentration=0.5 g/l.

Table 4.7. Effect of proteins on the activity of *Kluyveromyces lactis* β-galactosidase. All lactose solutions were prepared in 0.025 M monopotassium phosphate. The pH was adjusted to 6.8 with 5.0 M KOH. T=38°C.

	% LACTOSE HYDROLYSIS		
	TIME(min)		
Substrate	5	15	30
Control[1]	23.2±0.8	50.6±0.9	70.9±0.1
β-lg[2]	22.1±0.0	48.7±0.7	72.6±1.1
BSA[3]	22.0±0.4	47.8±0.2	70.4±0.7
Ovalbumin[4]	21.8±0.8	49.2±1.1	71.5±0.9

[1] 0.146 M lactose.
[2] 0.146 M lactose + 3 g/l β-lactoglobulin.
[3] 0.146 M lactose + 0.3 g/l bovine serum albumin.
[4] 0.146 M lactose + 5.0 g/l ovalbumin.

of β-lactoglobulin, bovine serum albumin and ovalbumin, respectively, in the same substrate at pH 6.6. It was not possible to reproduce their results in several repeated experiments. The effect of the whey proteins observed by Mahoney and Adamchuk (1980) may be more important for the stabilization of the neutral β-galactosidase when hydrolyzing sweet whey, where not as much neutralizing agent would be required.

The discrepancy between the results obtained here and those of Mahoney and Adamchuk (1980), as well as the explanation for the differences in hydrolysis rate in skim milk, whey, and UF permeate may again be related to the pH-adjusting step or to the different enzymes used. The amount of 5.0 M KOH required to bring the pH to 6.8 was different for each of the substrates. The pH of skim milk was 6.8 and did not need any adjustment. The amount of 5.0 M KOH required to bring the pH of 1 litre of whey from 4.6 to 6.8 was 5 ml, while only 3 ml were needed for the permeate. The buffering capacity of the whey proteins, present only in the whey, may be the reason for this difference. Therefore, after pH adjustment, the number of potassium ions in whey was higher than in UF permeate and in skim milk, and this was evident in a higher β-galactosidase activity.

4.2.4 Michaelis-Menten constant of *K. lactis* lactase.

The K_m obtained for the *K. lactis* enzyme was 76.9 mM. K_m values obtained for β-galactosidase preparations from other sources and with various degrees of purity have been published. Mahoney and Whitaker (1977) reported a K_m of 13.9 mM for a preparation from *Kluyveromyces fragilis* purified to electrophoretic, chromatographic and immunochemical homogeneity. Since these proteins are not the same, it should not be surprising that even closely related enzymes like the β-galactosidases from *K. lactis* and *K. fragilis* present differences in their affinity for lactose.

The purification of enzyme preparations could result in a decrease in K_m value if any possible inhibitor present in these preparations is removed. Working with an *Aspergillus niger* β-galactosidase purified from a commercial preparation by affinity chromatography, Woychik and Wondolowski (1972) reported a K_m value of 20.0 mM, while a value of 86.0 mM was found by Stanley *et al.*(1975) for a commercial preparation from the same source used without further purification.

The differences in the activity of the enzyme with the substrates considered in this work were likely determined by the nature and amount of neutralizing agent used and its interactions with the components of the substrates rather than by the proteins themselves. The higher enzymatic activity observed in whey due to the addition of potassium ions may be advantageous for hydrolyzing acid whey after neutralization, if the economics of this approach would be favourable.

4.3 Comparison of six commercial betagalactosidases for lactose hydrolysis in cottage cheese whey.

4.3.1 Introduction.

The hydrolysis of lactose in whey is the most promising alternative available for the utilization of the lactose portion of this dairy byproduct (Jelen, 1983). It might be of interest for the cheese producers to consider the manufacture of lactose hydrolyzed whey beverages in their plants. For the selection of the proper enzyme, objective and reliable information regarding the suitability of the commercial lactase preparations currently available for use in cottage cheese whey is necessary. The amount and cost of enzyme needed, the reaction time and conditions, and the final added value of the product obtained are some important factors that must be taken into account when considering the commercial use of enzymes in the food industry.

The main objective of this part of the work was to compare several commercially available lactase preparations with the aim of selecting the best alternative for the hydrolysis of lactose in cottage cheese whey. Two approaches were taken for this comparison. In the first one, the enzymes were compared on the basis of mathematical relationships derived from-steady state kinetic parameters obtained in model lactose solutions. In the second approach, the comparison was made on the basis of the progress curves obtained in cottage cheese whey for each enzyme at different concentrations.

4.3.2 Mathematical expressions derived from the Michaelis-Menten kinetic model.

Recently, Fullbrook (1983) presented some modifications to the classical kinetic models, pointing out their usefulness for the prediction of enzyme efficiencies in carrying out substrate conversions in industrial situations. This new approach was compared with a more empirical method where the progress curves for the different enzymes in the actual industrial hydrolysis conditions are used as basis of comparison. It was considered justifiable to include the mathematical development of the parameters used in the first approach to compare the enzymes, as proposed by Fullbrook (1983).

Henri (in 1903) and Michaelis and Menten (in 1913) developed an equation describing the dependence of the initial velocity (v) of an enzymatic reaction on substrate concentration (S):

$$v= \frac{Vmax\ S}{K_m + S} \qquad (1)$$

K_m, the Michaelis Menten constant, is the value of substrate concentration which gives an initial velocity equal to half the maximum velocity (*V*max) at a given enzyme concentration (E). This K_m is independent from E, is a true characteristic of the enzyme, and is a measure of its affinity for a particular substrate; a low K_m representing a high affinity for the substrate and vice versa. The simplest way of comparing several enzymes may be to look at their K_m.

Vmax is attained when all the enzyme molecules are saturated with substrate and is given by the expression:

$$V\text{max} = k\text{cat} \cdot E \qquad (2),$$

where $k$cat, or turnover number, is defined as the first order rate constant for the breakdown of the enzyme/substrate complex, and is related to the number of moles of substrate converted by the enzyme per unit time. If the enzymes were saturated with substrate, the fastest conversion per unit of enzyme activity would be given by the enzyme with the highest $k$cat. In industrial situations, however, the reactions may take place at low S/K_m ratios, and not only $k$cat but also K_m and S should be taken into account.

One approach to compare the efficiencies of several enzymes is the so called Physiological Efficiency (P.E.) concept (Fullbrook, 1983). P.E. is defined as the ratio $V\text{max}/K_m$ for a given amount of enzyme. By including Vmax, this concept incorporates $k$cat and E:

$$\text{P.E.} = \frac{V\text{max}}{K_m} = \frac{k\text{cat} \cdot E}{K_m} \qquad (3)$$

Since a comparison should be made at similar enzyme concentrations for all the enzymes analyzed at one time, the ratio $k\text{cat}/K_m$ can be used for this purpose.

The enzyme concentration and the reaction time necessary to effect a desired conversion yield can be obtained by combining equations (1) and (2) to obtain:

$$v = \frac{kcat \cdot E}{1 + (K_m/S)} \qquad (4)$$

Furthermore, if

$$v = ds/dt \qquad (5),$$

then,

$$kcat\ (E)\ dt = (K_m/S)\ ds + ds \qquad (6)$$

and, integrating and solving for t , we obtain

$$t = \frac{(S_0 - S) + K_m \ln (S_0/S)}{kcat \cdot E} \qquad (7)$$

The reaction time (t) required to change the substrate from an initial concentration (S_0) to a given concentration (S), for a given E, is related by the constants K_m and $k$cat when the enzyme remains at constant activity throughout the reaction. If conversion is defined as:

$$X = \frac{(S_0 - S)}{S} \qquad (8)$$

equation (7) becomes

$$t = \frac{S_0 X + K_m \ln (1/(1-X))}{E \cdot kcat} \qquad (9)$$

Equation (9) can be used to calculate the minimum level of enzyme required to effect a given level of conversion of substrate from an initial concentration and in a finite time. This minimal enzyme dosage would be the actual amount of enzyme required only if the enzyme would maintain its full activity during the whole course of the reaction; however, this is very unlikely to happen.

Combining with (2) and replacing the limits for the integration of equation (6) with $(S,S_{1/2})$ and $(t,t_{1/2})$ instead of (S,S_0) and (t,t_0), the equation (10) is obtained:

$$t_{1/2} = \ln 2 \; \frac{K_m}{Vmax} + \frac{S}{2\;Vmax} \qquad (10),$$

where $t_{1/2}$ would be the time it takes for the enzyme to reduce the substrate level to half its original concentration. The lower this $t_{1/2}$ value, the higher the efficiency of the enzyme.

4.3.3 Kinetic parameters of the commercial β-galactosidase preparations and their comparison.

For each of the six commercial preparations, K_m and *V*max were determined from initial velocity measurements at two different enzyme concentrations. For the two neutral preparations (A and B) these parameters were determined with and without potassium (Table 4.8). The results indicate that although the two enzymes may be closely related, their response to potassium activation is quite different. The values obtained in the presence of 0.025 M monopotassium phosphate were the ones used for all the calculations.

The parameters obtained from the kinetic study of the enzymes are given in Table 4.9. By comparing their K_m, the best enzymes would seem those with the lowest $K_m$values (products B, E and D). If all the enzymes were saturated

Table 4.8. Effect of 0.025 M KH_2PO_4 on the kinetic parameters obtained for the neutral β-galactosidase commercial preparations. Enzyme concentration=1g/l.

KINETIC PARAMETERS OF THE NEUTRAL β-GALACTOSIDASE PREPARATIONS.

ENZYME	K_m[1]	*V*max[2]
A	156.0	22.5
A in 0.025M KH_2PO_4	76.8	22.5
B	24.2	56.7
B in 0.025M KH_2PO_4	24.2	78.3

[1] mM.

[2] mM/g s.

Table 4.9. Kinetic derived parameters of comparison for the six commercial enzyme preparations of β-galactosidase.

ENZYME	K_m[1]	*k*cat[2]	P.E.[3]	t[4]	$t_{1/2}$[5]
A	76.8	4.73	0.0616	28.62	12.75
B	24.2	3.88	0.1603	13.05	6.18
C	101.8	27.11	0.2663	6.47	2.86
D	68.5	19.99	0.2918	6.10	2.73
E	50.6	17.99	0.3555	5.18	2.35
F	125.2	16.37	0.1308	17.25	5.73

---

[1] mM.
[2] Turnover number.
[3] Physiological efficiency(Vmax/K_m).
[4] Time required for an 80% hydrolysis.
[5] Time required for the enzyme to reduce the lactose concentration to half its original level.

with substrate to the same extent, the fastest conversion rate would be given by enzyme C, the enzyme with the highest Kcat value.

Using P.E. as the index of comparison, enzymes E, D and then C would be the best options. However, if the substrate concentration was much higher than the K_m, P.E. values would be of limited use.

Both the reaction times required to hydrolyze 80% of the initial lactose and the half time values also indicate that enzymes E, D and C would be the best choices among the commercial preparations compared. It should be remembered that these times would be only the minimum required times, because it was assumed for the calculation that the activity of the enzymes would remain unchanged during the reaction.

Several enzymes were pointed out as the best alternatives by using the different parameters, creating confusion about which of the parameters should be taken as the most reliable or accurate index of comparison. It would seem that enzyme E would be the best alternative, because this enzyme was first in three out of the five parameters used in the comparison. However, it does not seem sensible to select this or any other enzyme based on this method only, in view of the different results obtained and before having compared their activities under the actual hydrolysis conditions in cottage cheese whey.

In general, this uncertainty appears to be a big disadvantage for this method in the present conditions. As will be shown in the following sections, the "traditional" empirical approach may have to be used as the only alternative available for the comparison of industrial enzyme preparations.

4.3.4 Empirical comparison of the commercial preparations on the basis of their activity in cottage cheese whey.

The empirical approach involved the direct comparison of the progress curves of each enzyme at several concentrations. These progress curves are given in Figures 4.4 to 4.15. At all the concentrations studied, enzyme C had the highest activity of all six β-galactosidase preparations and therefore it would be the best alternative among the enzymes compared by this empirical method.

Enzyme C was also pointed out as the best preparation when the $k$cat values of the preparations were used as the determinant in the kinetic-based approach (Table 4.8). However, $k$cat cannot be taken as a dependable index of the activity of one preparation against the others since the concentration of lactose indicates that the six enzymes, particulary those with the higher K_m values (enzymes C and F), will not be saturated to the same extent when carrying out the hydrolysis of lactose in whey. From the examination of these progress curves (Figures 4.4 to 4.15) it seems that, based in the criteron of efficiency, the best choice would be enzyme C, even if this enzyme was not pointed out to be the one with the highest overall potential by the

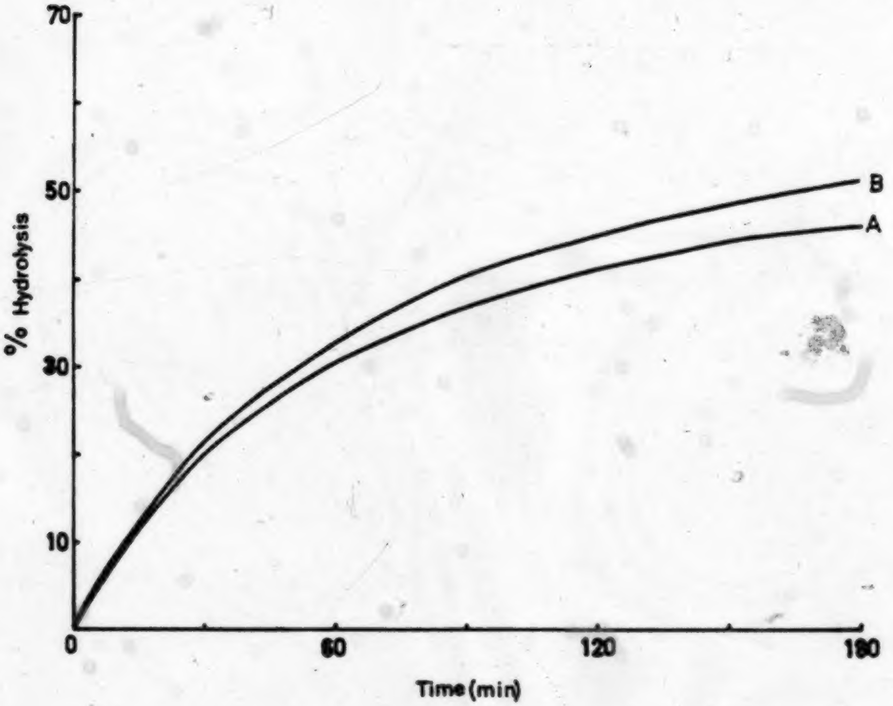

Figure 4.4. Progress curves for the hydrolysis of lactose in whey using neutral lactases. Enzyme concentration=0.1 g/l, T=38°C and pH=6.8.

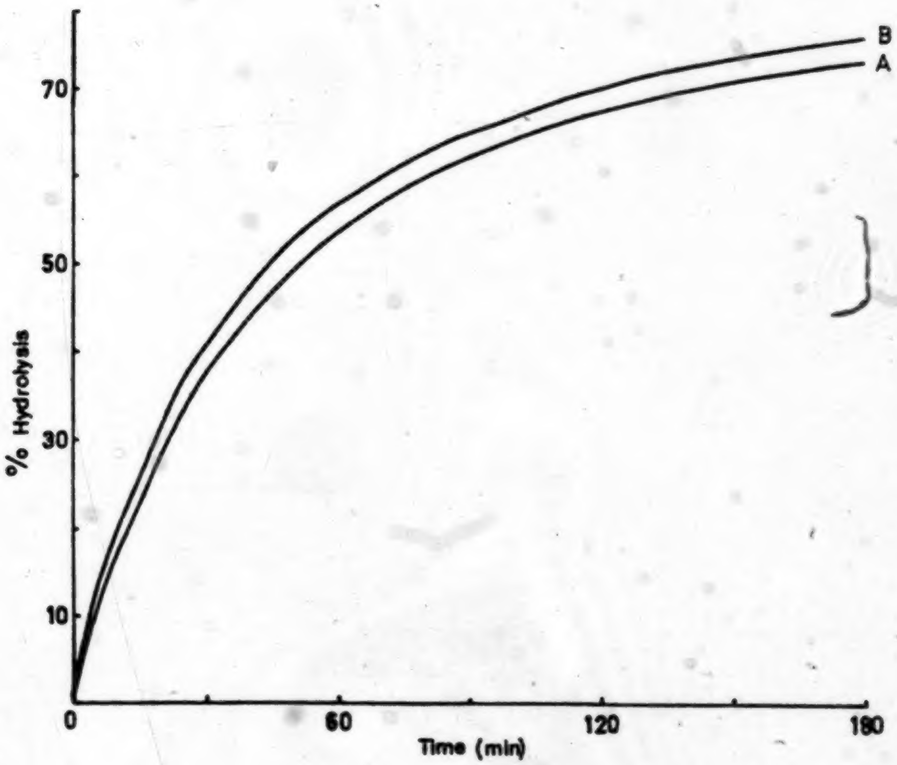

Figure 4.5. Progress curves for the hydrolysis of lactose in whey using neutral lactases. Enzyme concentration=0.3 g/l, T=38°C and pH=6.8

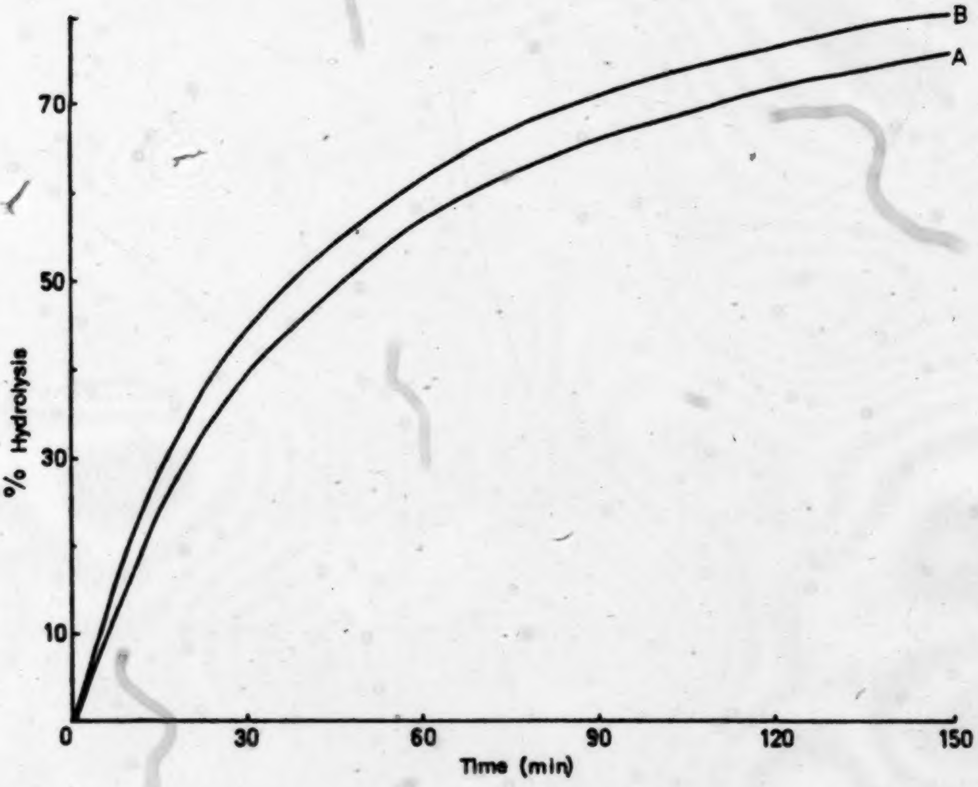

Figure 4.6. Progress curves for the hydrolysis of lactose in whey using neutral lactases. Enzyme concentration=0.5 g/l, T=38°C and pH=6.8.

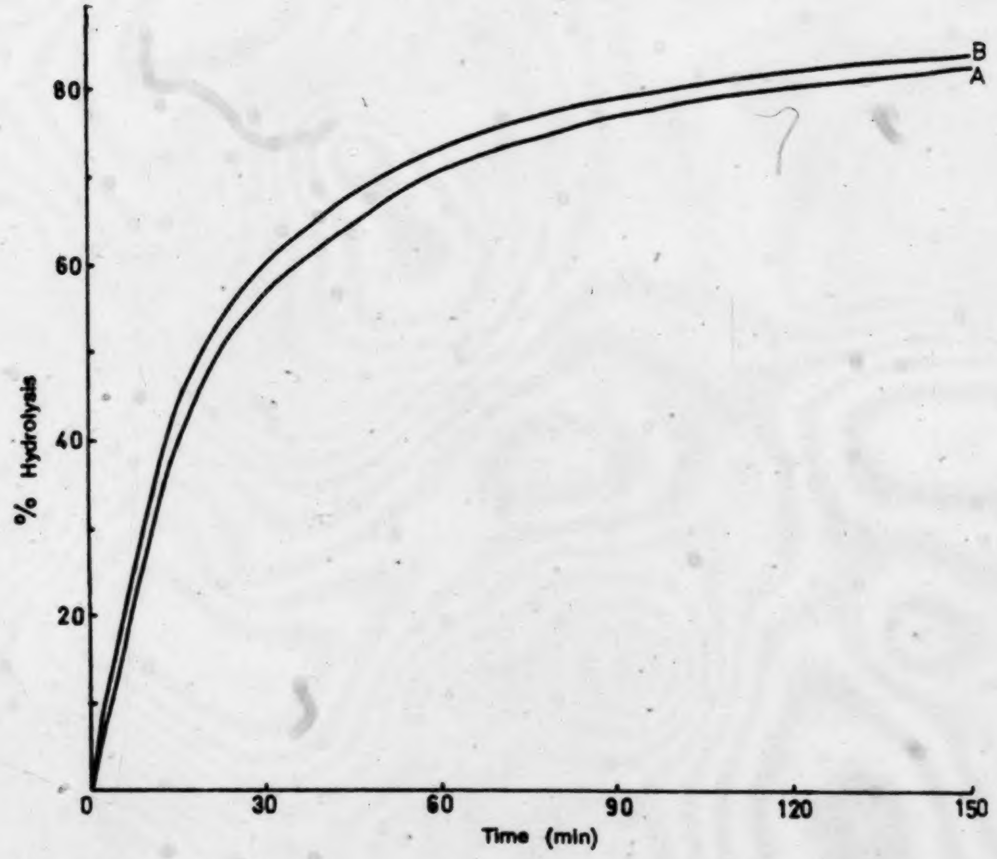

Figure 4.7. Progress curves for the hydrolysis of lactose in whey using neutral lactases. Enzyme concentration=1.0 g/l, T=38°C and pH=6.8.

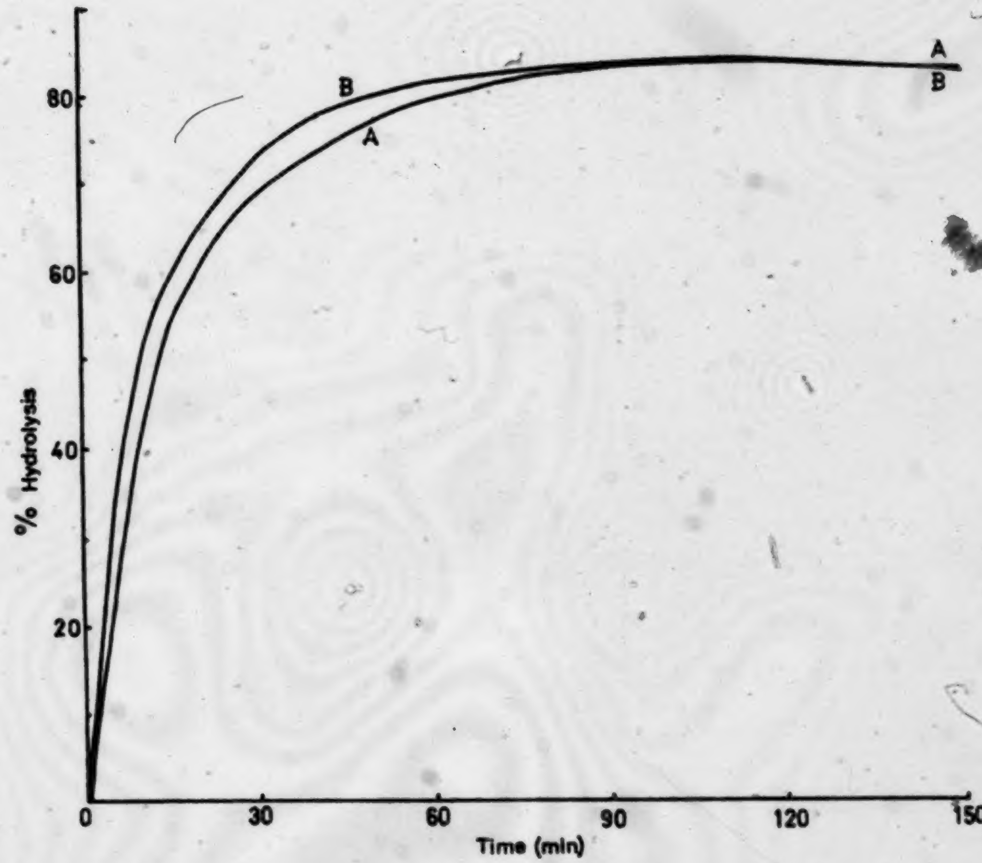

Figure 4.8. Progress curves for the hydrolysis of lactose in whey using neutral lactases. Enzyme concentration=2.0 g/l, T=38°C and pH=6.8.

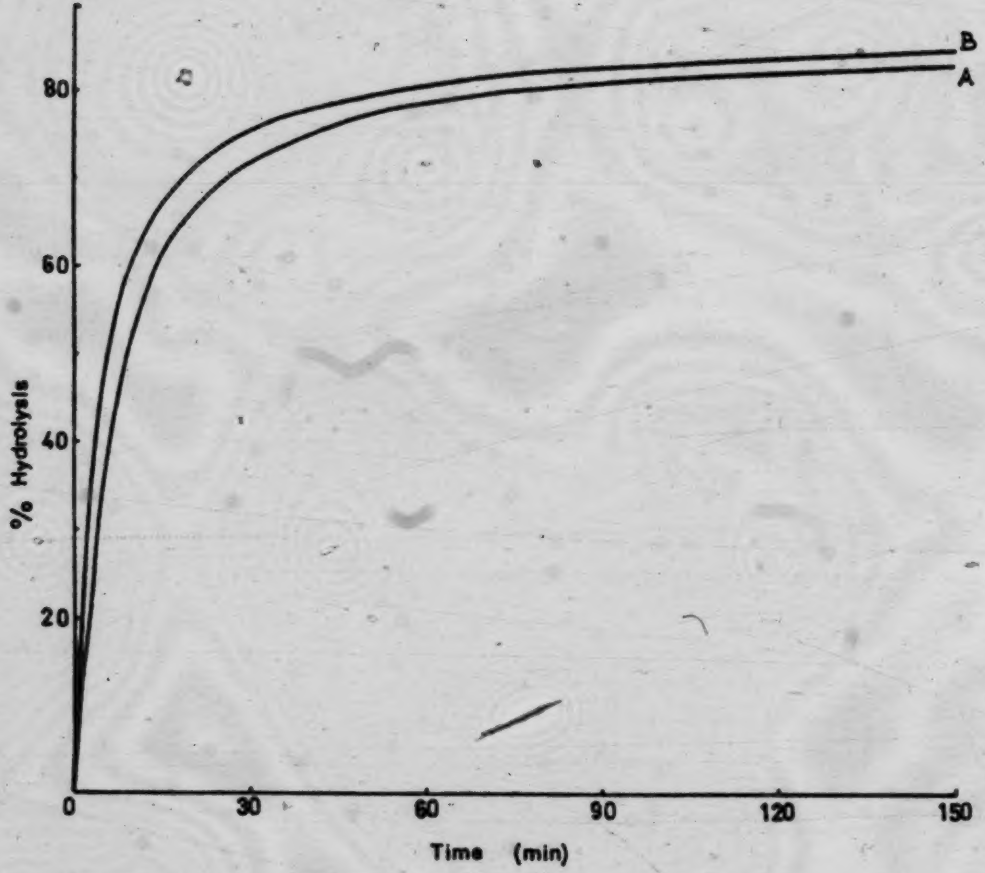

Figure 4.9. Progress curves for the hydrolysis of lactose in whey using neutral lactases. Enzyme concentration=3.0 g/l, T=38°C and pH=6.8.

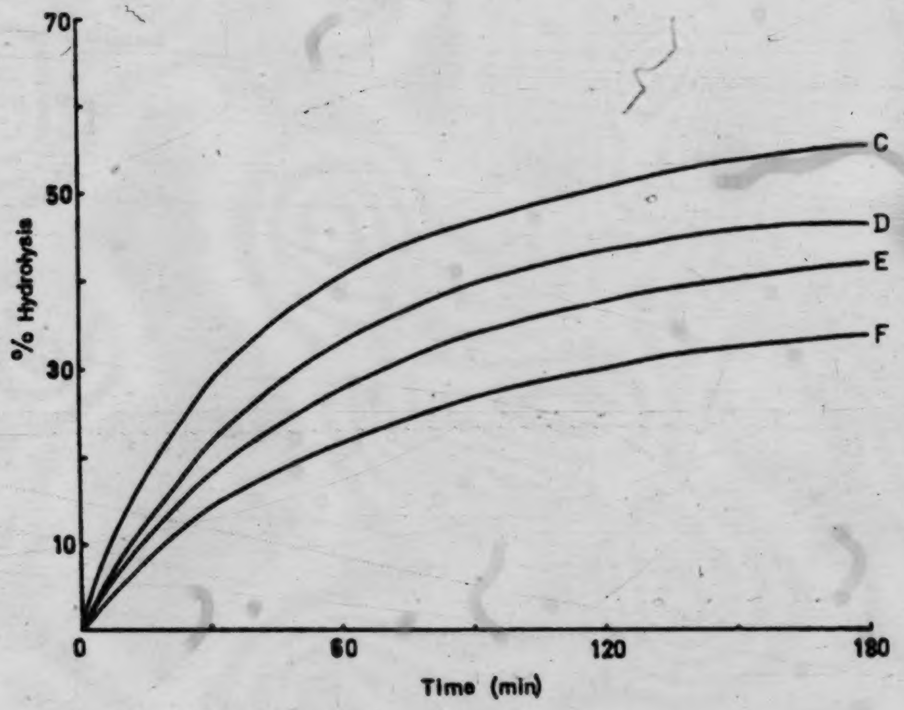

Figure 4.10. Progress curves for the hydrolysis of lactose in whey using four acid lactases. Enzyme concentration=0.1 g/l, T=55°C and pH=4.6.

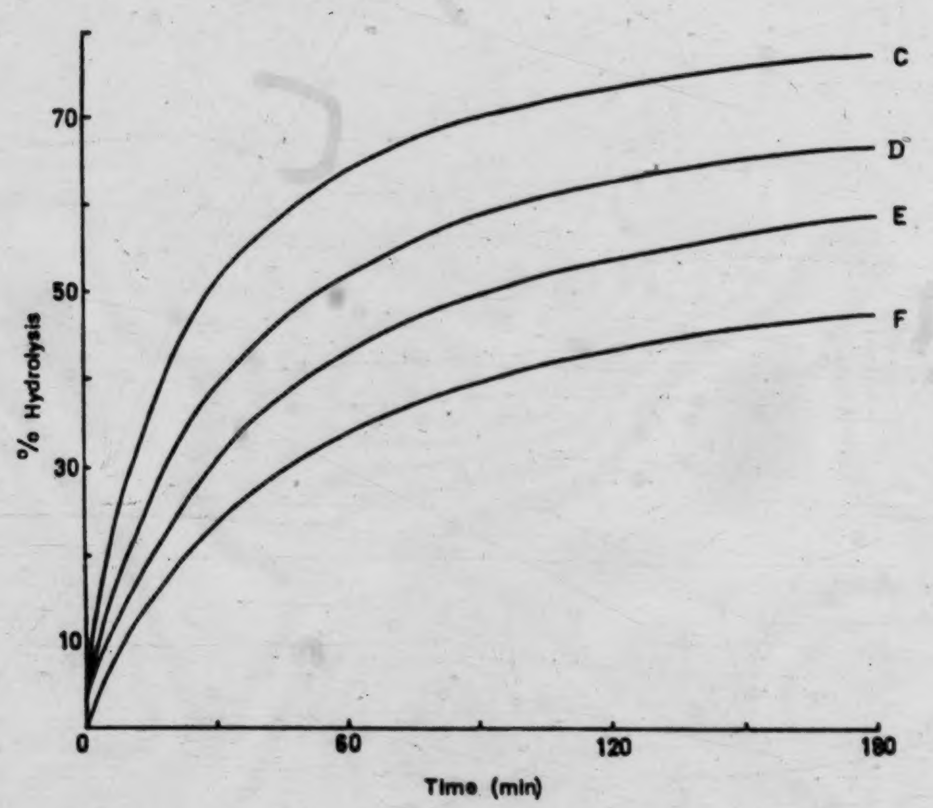

Figure 4.11. Progress curves for the hydrolysis of lactose in whey using acid lactases. Enzyme concentration=0.3 g/l, T=55°C and pH=4.6.

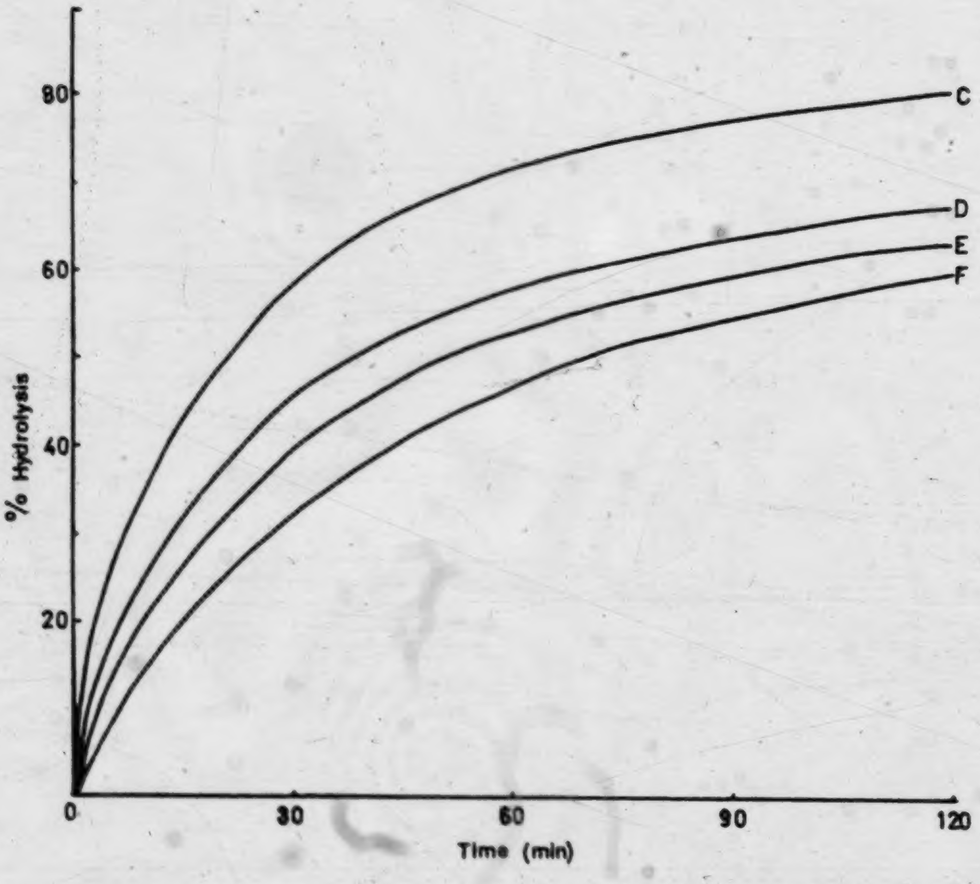

Figure 4.12. Progress curves for the hydrolysis of lactose in whey using acid lactases. Enzyme concentration=0.5 g/l, T=55°C and pH=4.6.

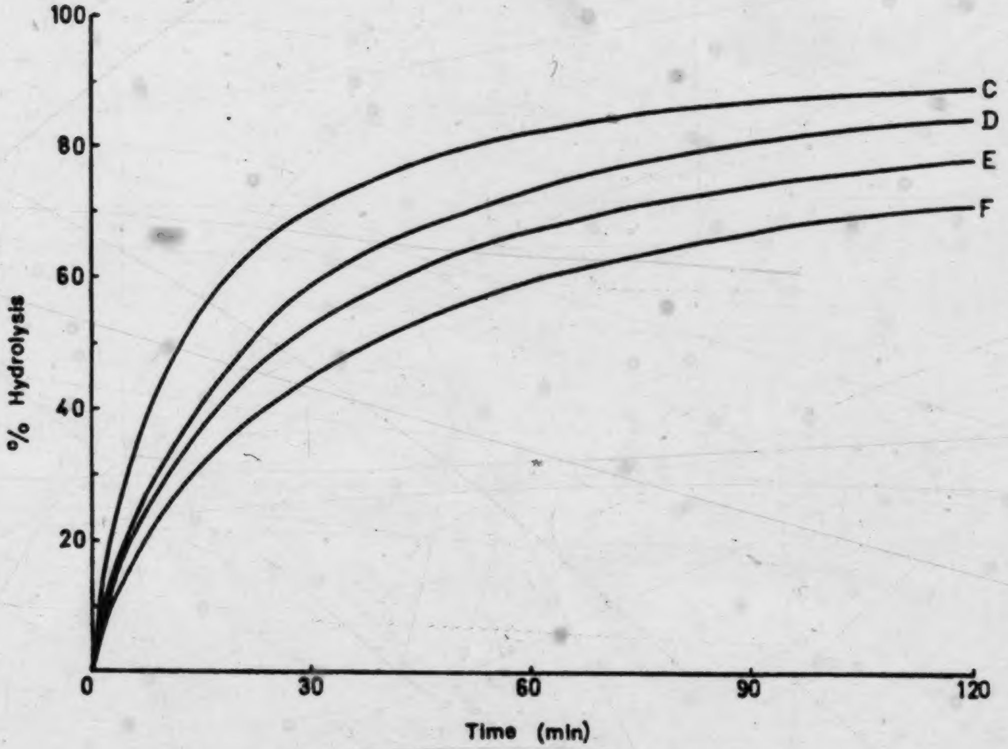

Figure 4.13. Progress curves for the hydrolysis of lactose in whey using acid lactases. Enzyme concentration=1.0 g/l, T=55°C and pH=4.6.

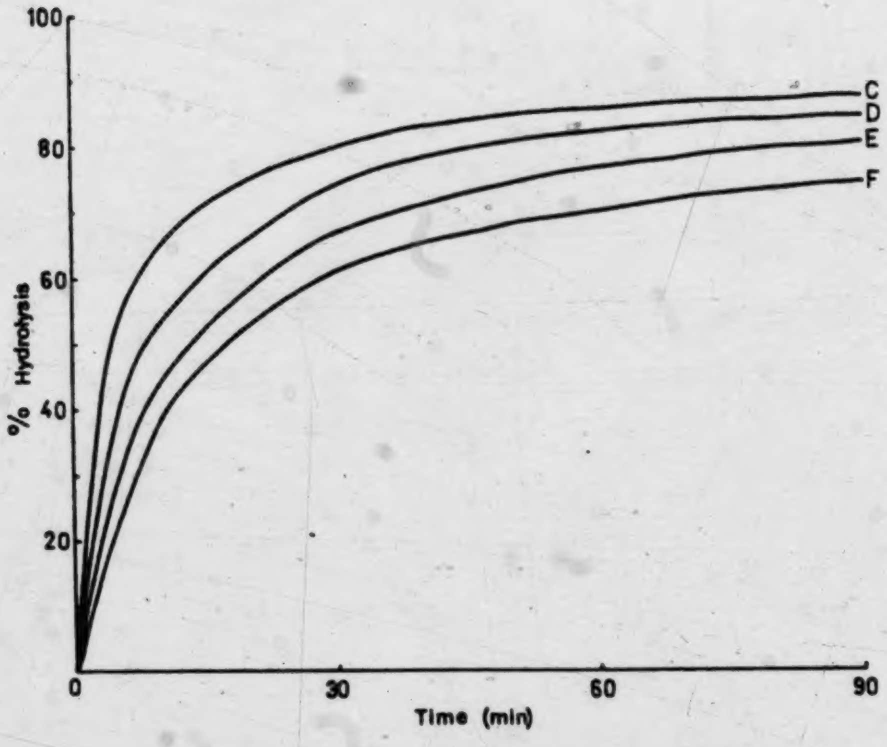

Figure 4.14. Progress curves for the hydrolysis of lactose in whey using acid lactases. Enzyme concentration=2.0 g/l, T=55°C and pH=4.6.

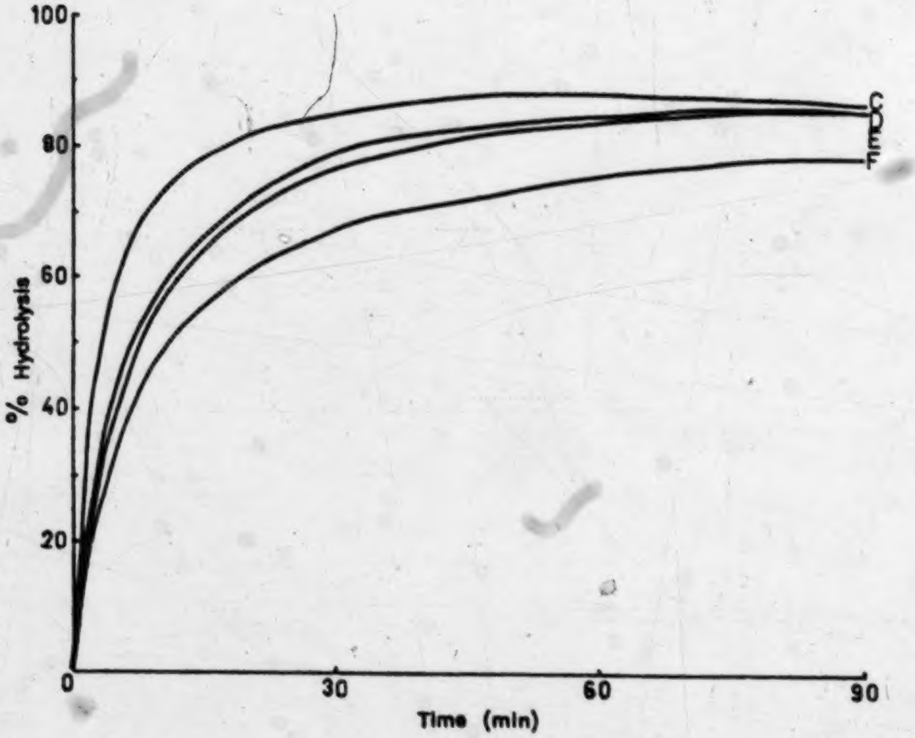

Figure 4.15. Progress curves for the hydrolysis of lactose in whey using acid lactases. Enzyme concentration=3.0 g/l, T=55°C and pH=4.6.

kinetic-derived approach.

Figures 4.16 to 4.21 were built from the data shown in the progress curves, and therefore they also point out enzyme C as the best alternative. These figures present the enzyme concentration/hydrolysis time combinations required to obtain an 80% hydrolysis in whey and permit the best selection of the enzyme/time compromise for any particular processing situation and for any of the six preparations included in the present study.

4.3.5 Galactose inhibition.

The inhibitory effect of galactose on the activity of the neutral β-galactosidases considered in this study is very well known and has been studied extensively (Fullbrook, 1983; Burguess and Shaw, 1983). On the other hand, product inhibition by galactose of the acid *Aspergillus oryzae* β-galactosidase has been claimed to be less pronounced (Sprossler and Plainer, 1983). This decreased sensitivity to product inhibition was pointed out by the same authors as an important advantage for the *A. oryzae* β-galactosidase. However, Figures 4.22 to 4.25 show that the activity of the *A. oryzae* β-galactosidase was considerably reduced in the presence of galactose and therefore, galactose inhibition is still a problem with the use of these enzymes. The effect of galactose was concentration dependent as shown for the enzyme C in Figure 4.26. Galactose inhibition prevents the enzyme from hydrolyzing all of the lactose; therefore,

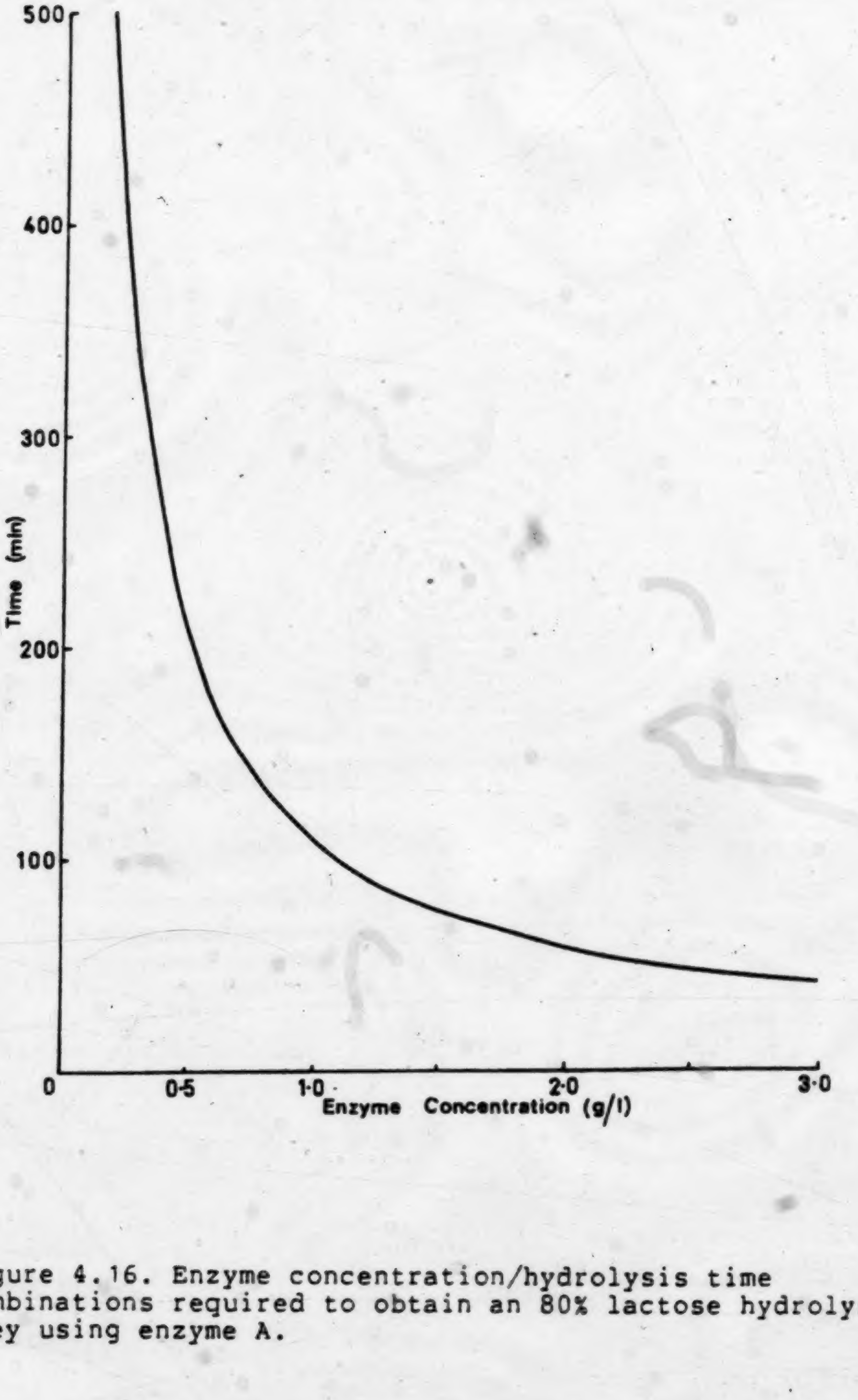

Figure 4.16. Enzyme concentration/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using enzyme A.

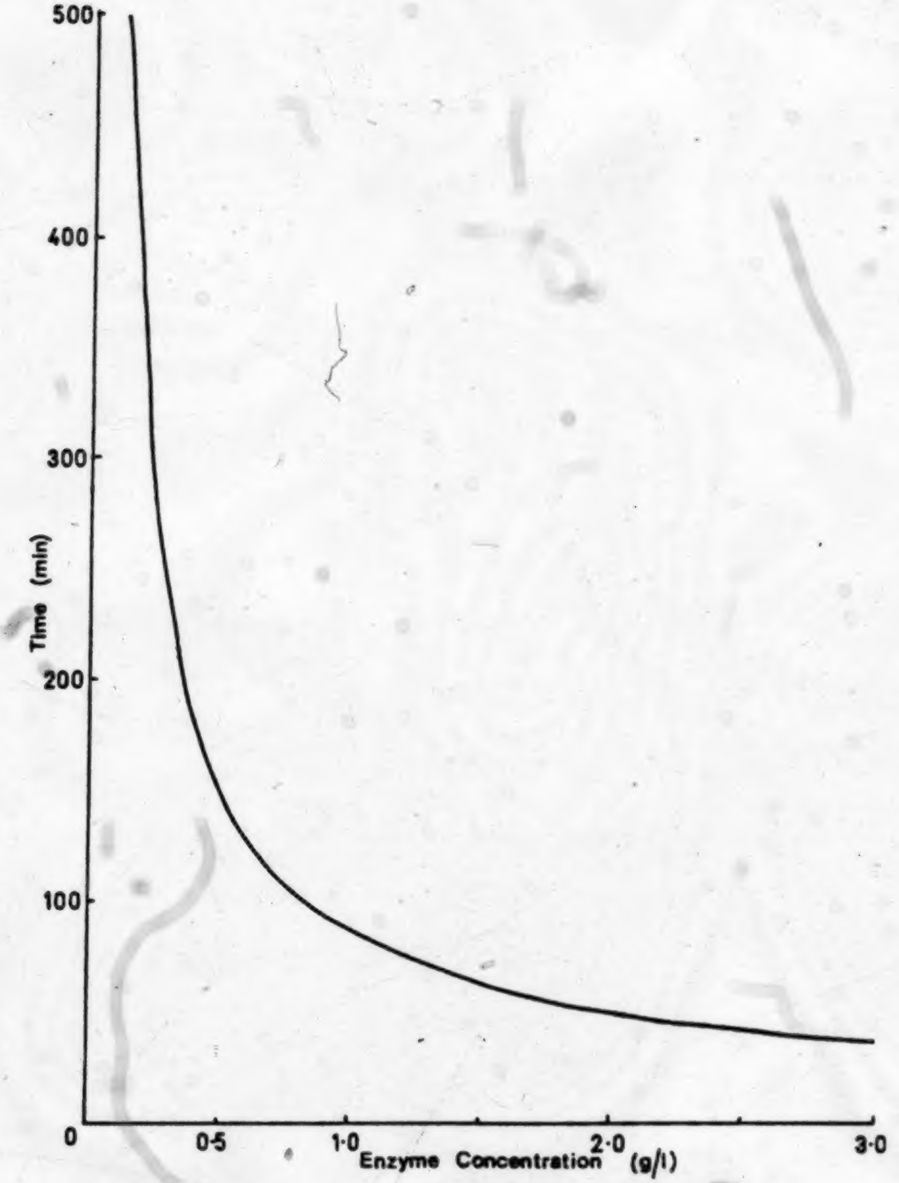

Figure 4.17. Enzyme concentration/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using enzyme B.

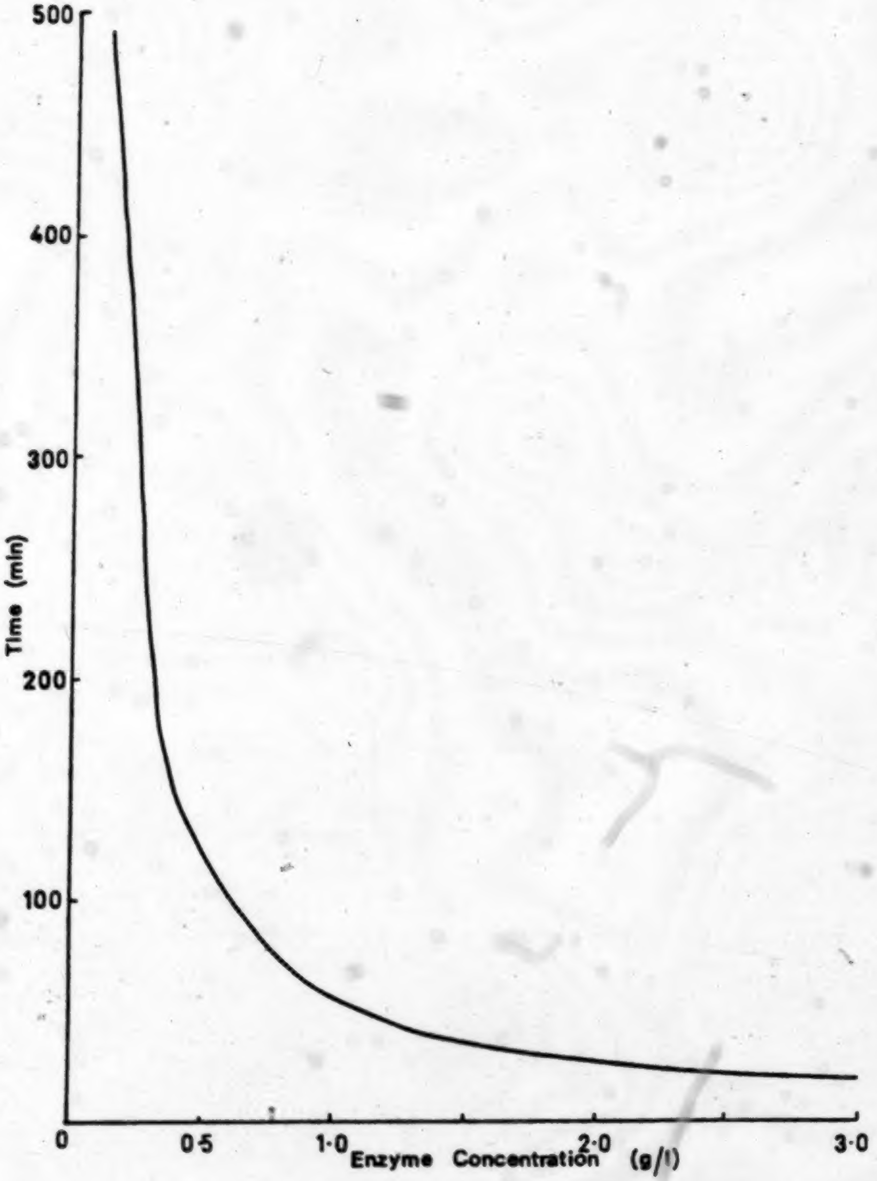

Figure 4.18. Enzyme concentration/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using enzyme C.

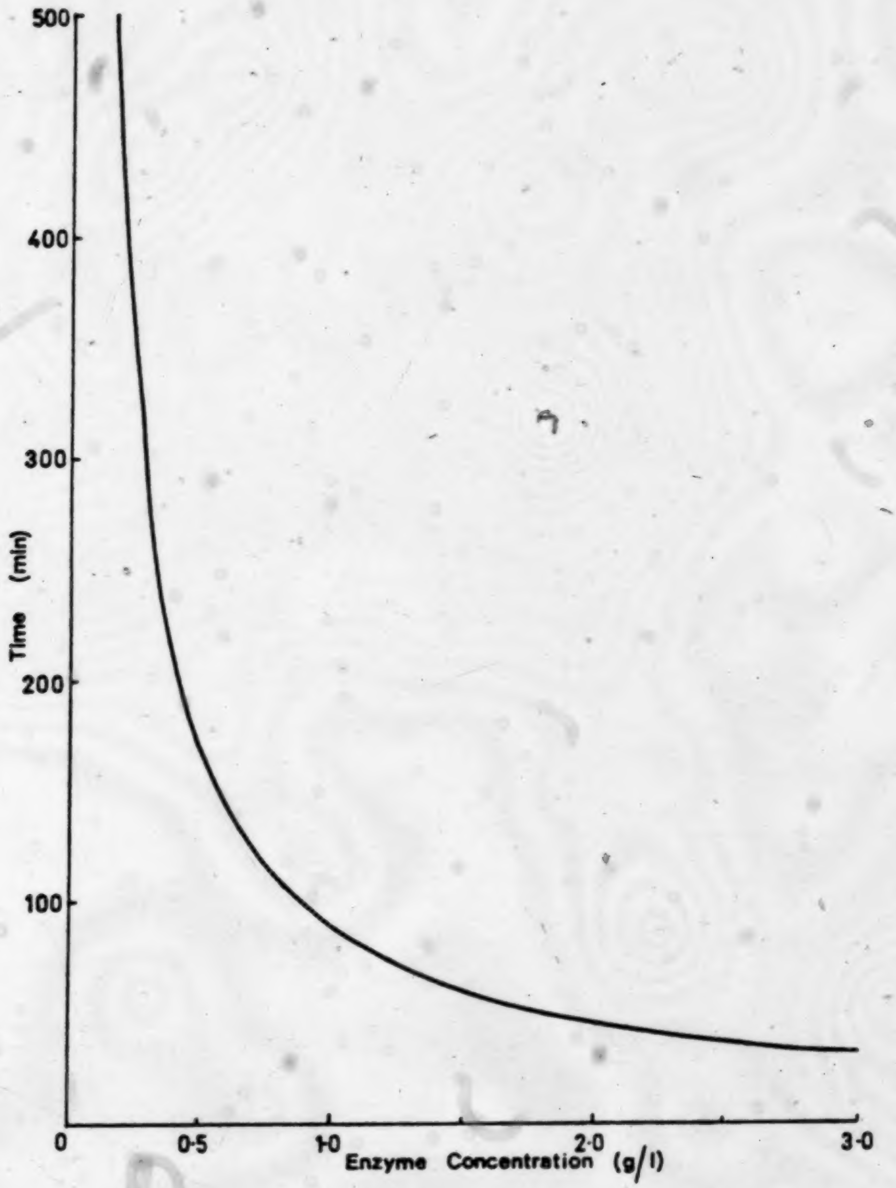

Figure 4.19. Enzyme concentration/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using enzyme D.

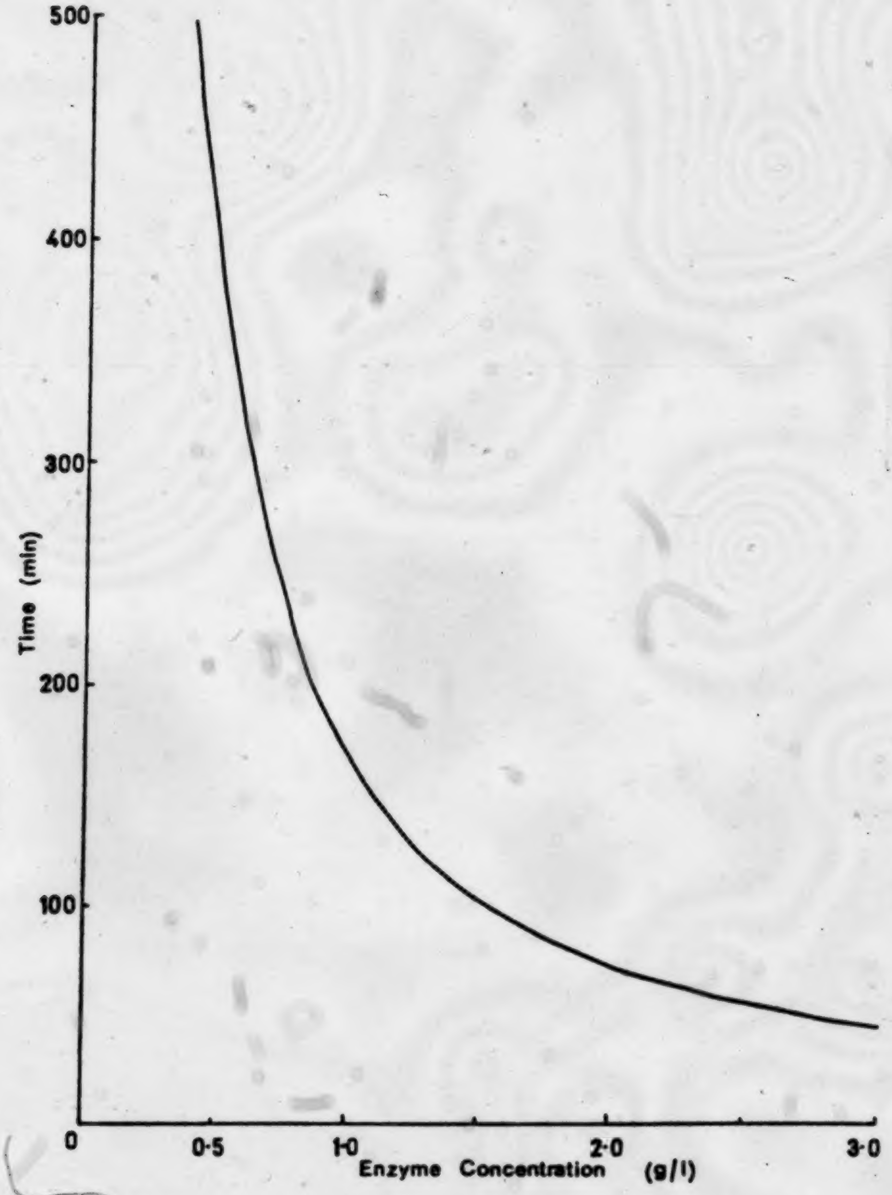

Figure 4.20. Enzyme concentration/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using enzyme E.

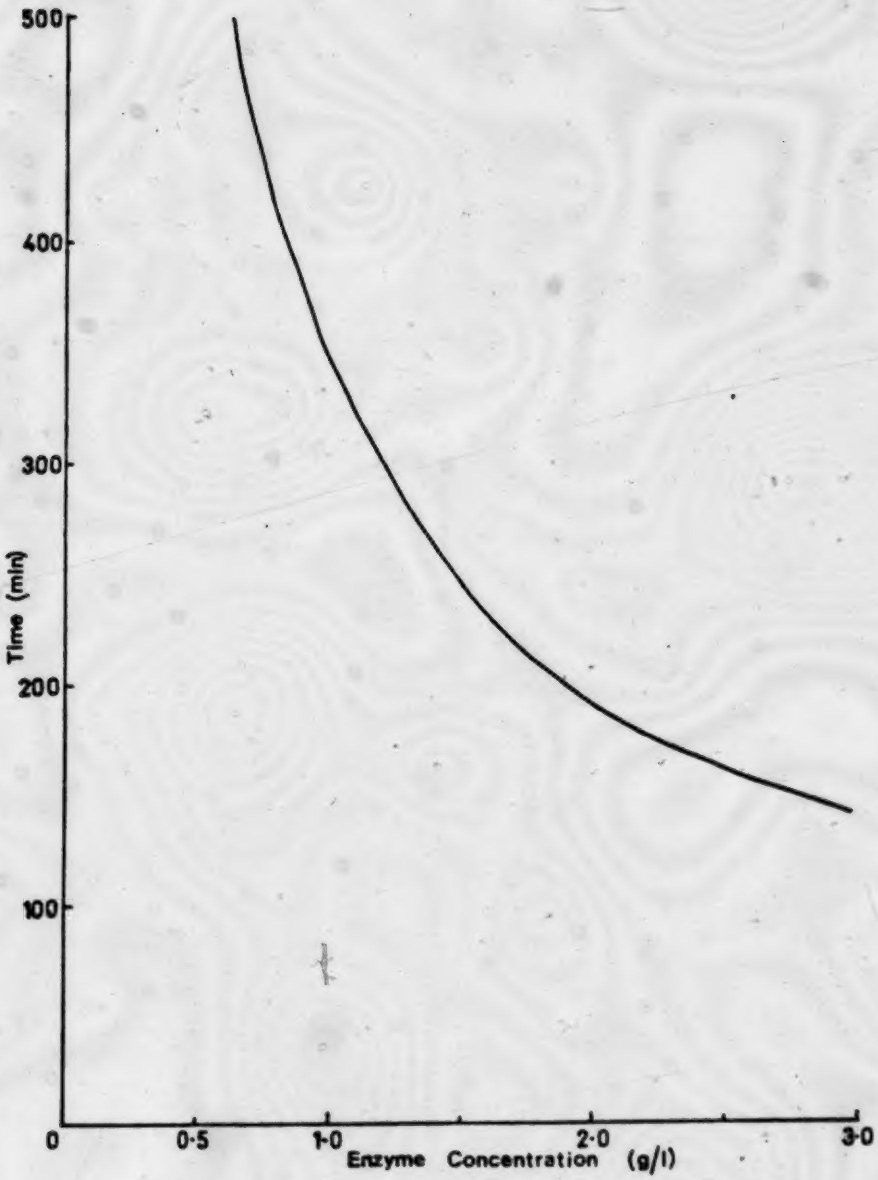

Figure 4.21. Enzyme concentration/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using enzyme F.

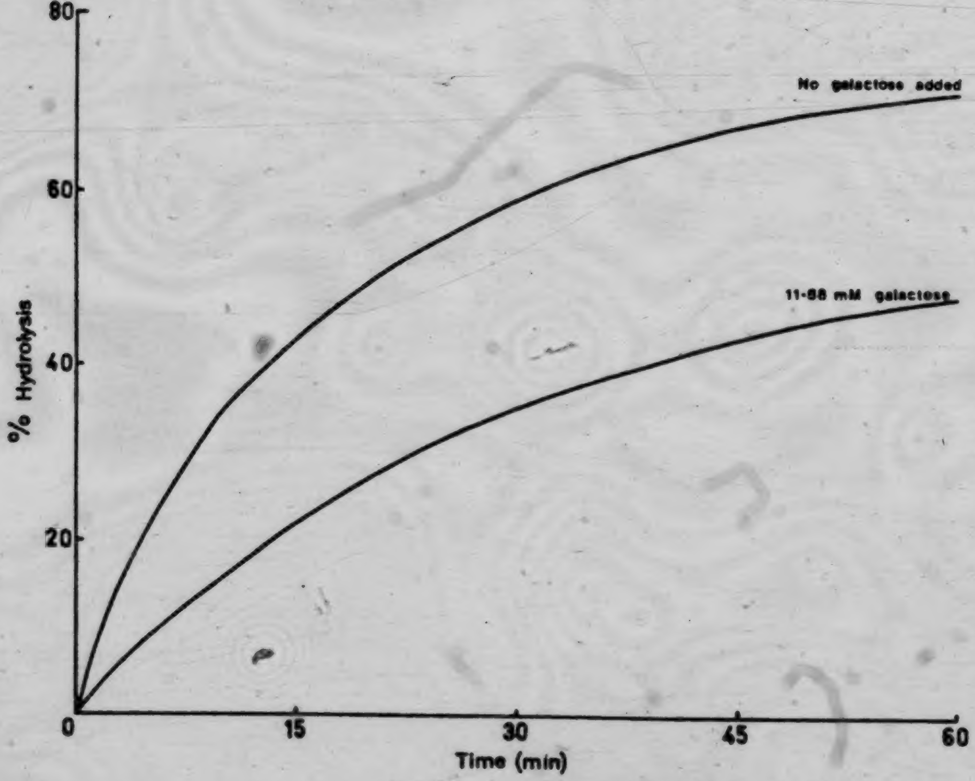

Figure 4.22. Galactose inhibition of the hydrolysis of lactose in cottage cheese whey using enzyme C. Enzyme concentration=0.5 g/l.

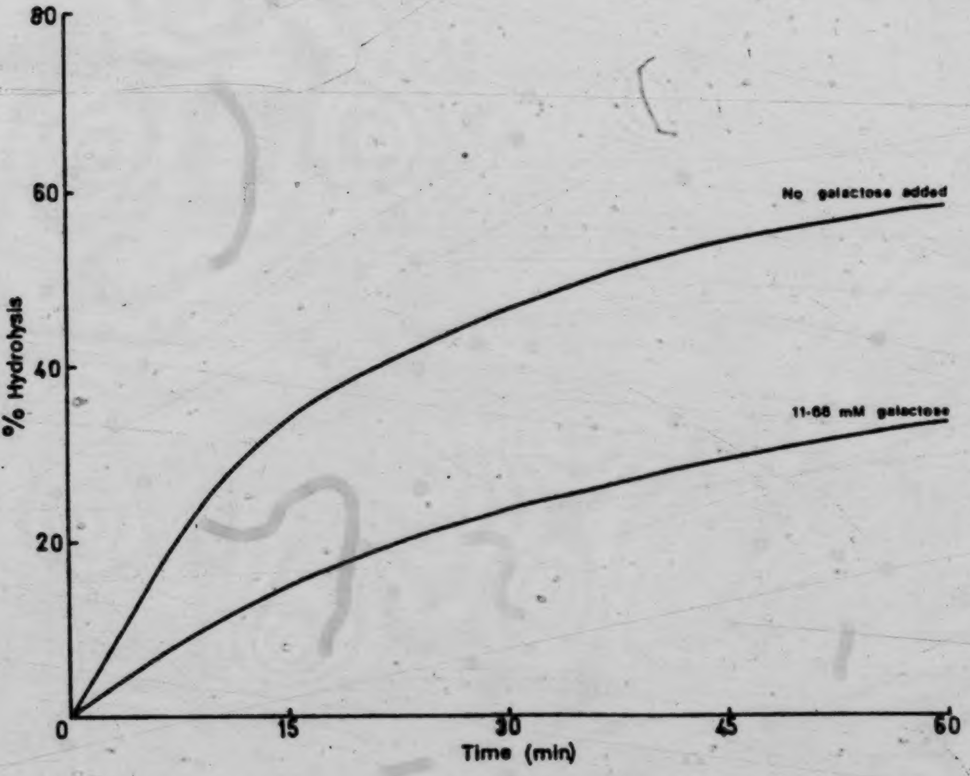

Figure 4.23. Galactose inhibition of the hydrolysis of lactose in cottage cheese whey using enzyme D. Enzyme concentration=0.5 g/l.

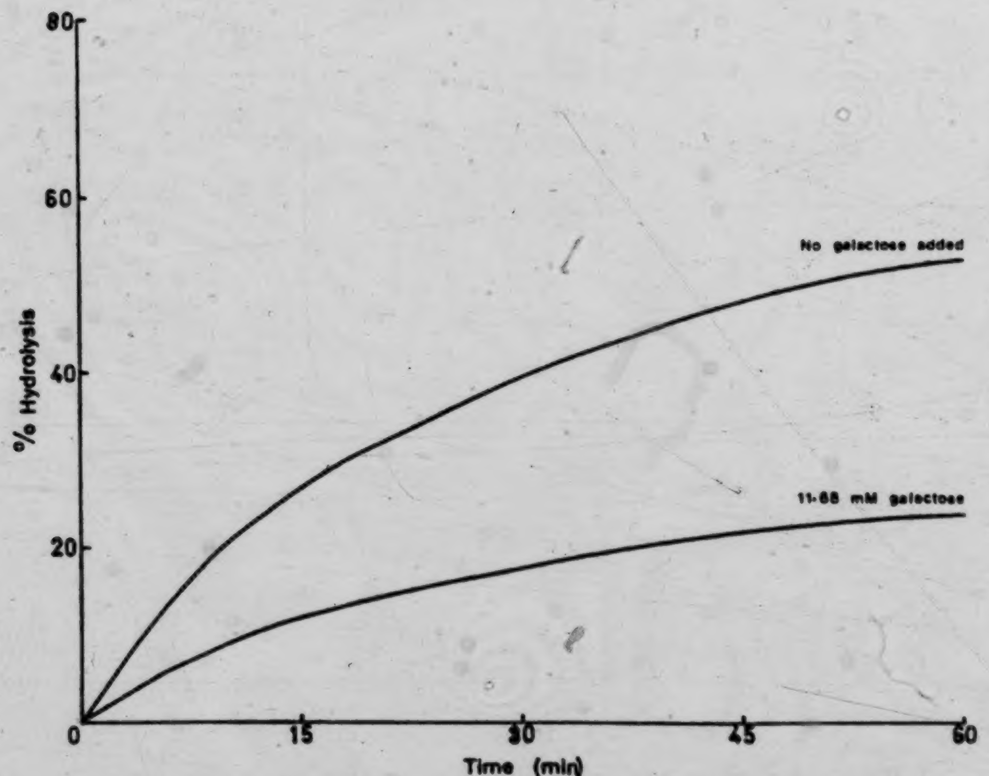

Figure 4.24. Galactose inhibition of the hydrolysis of lactose in cottage cheese whey using enzyme E. Enzyme concentration=0.5 g/l.

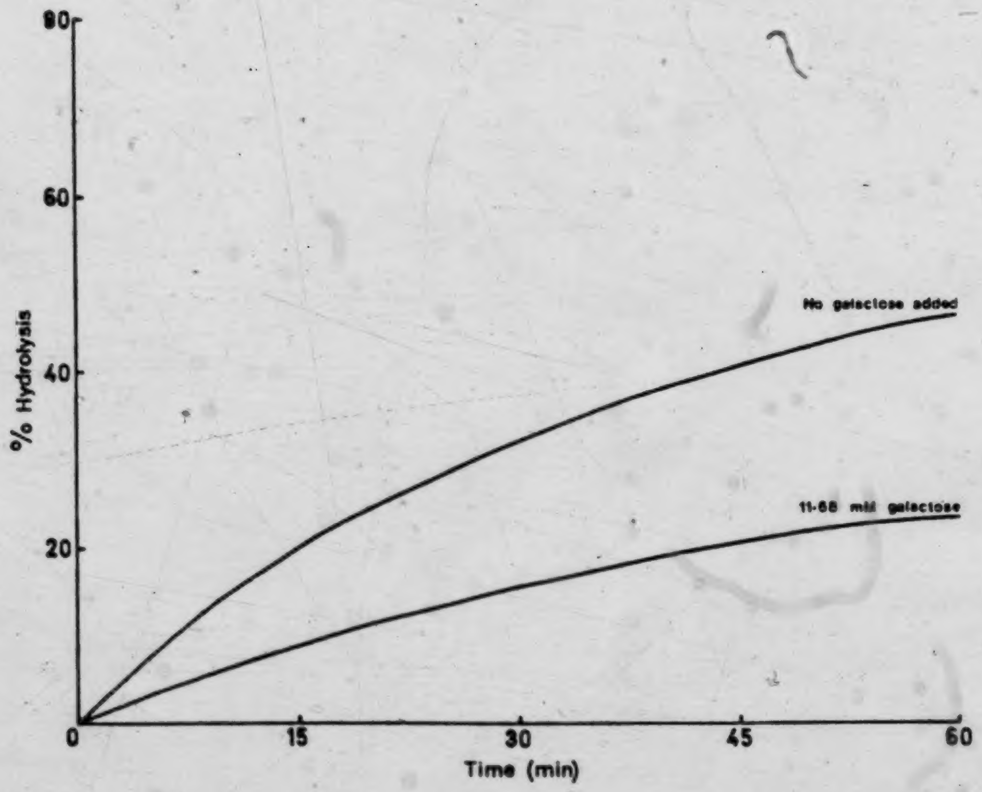

Figure 4.25. Galactose inhibition of the hydrolysis of lactose in cottage cheese whey using enzyme F. Enzyme concentration=0.5 g/l.

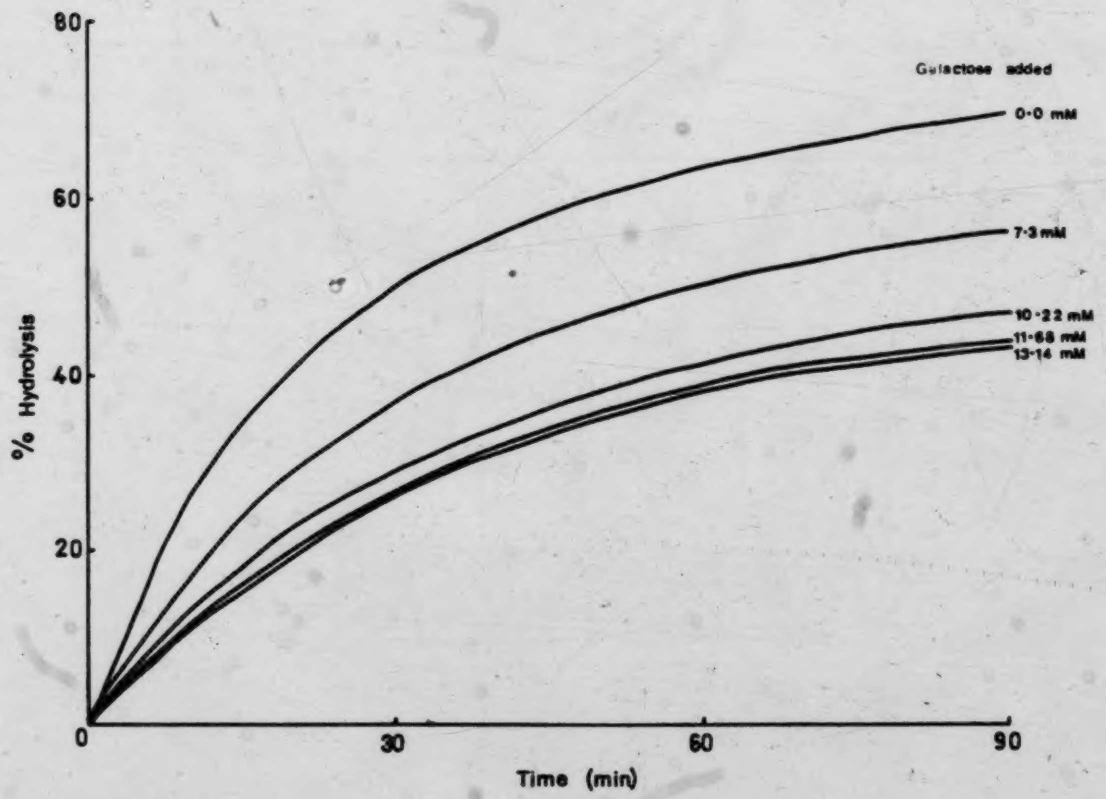

Figure 4.26. Influence of galactose concentration on the product inhibition observed for the lactose hydrolysis in whey using enzyme C.

aiming at 100% conversion in the final product is not very practical. Furthermore, the improvement of important functional properties has been observed when 20% of the lactose is left unhydrolyzed (Shah and Nickerson, 1978a; Shah and Nickerson, 1978b; Shah and Nickerson, 1978c).

4.3.6 Differences between the kinetic-based and the empirical approaches.

The empirical approach showed that enzyme C had the highest activity in cottage cheese whey. On the other hand, from the results obtained in the kinetic based comparison, it was more reasonable to expect enzyme E to have the best performance in whey. At the end, the prediction of the kinetic-based method proved to be inaccurate and therefore, the results given by the empirical comparison were taken as more reliable. Several reasons could account for the inaccuracy of the first approach.

For the kinetic-based parameters to be a good index of comparison, the enzymes should be all saturated with substrate. The concentration of lactose in whey (146 mM) indicates that there will be differences in the degree of saturation of the different enzymes during the hydrolysis of lactose in whey and therefore, some of the values used in the first approach would not give an accurate prediction of the enzyme performances observed in the second approach.

There were compositional differences between the lactose solutions used for the determination of the kinetic parameters and the whey with which the progress curves were obtained. Although the hydrolysis conditions (i.e. pH and temperature) were the same for both experiments, some of the whey constituents (in particular the whey proteins and minerals) were not present in the model lactose solutions where the kinetic parameters were determined.

It has been shown that when acid whey was neutralized with KOH, the activity of a *Kluyveromyces lactis* lactase was governed by the ionic environment rather than by any possible activating or inhibitory effect of the whey proteins (Bernal and Jelen, 1984b). Although the acid lactases have been reported to be insensitive to cation activation or inhibition (Sprossler and Plainer, 1983), the influence of other whey constituents should be established before any definitive conclusions can be made in this regard.

Possible differences in the sensitivity of the various enzymes to galactose inhibition and in the rate of gradual decrease in activity during the course of the reaction are two important factors that could not be estimated in the kinetic studies. These factors may become crucial when considering extended hydrolysis times in cottage cheese whey.

A very important advantage of the second (empirical) approach over the kinetic-based one is that the former, apart from providing a true comparison of the enzymes in the actual substrate of industrial relevance, permits the inclusion of the cost of the enzyme preparations into the comparison. An estimation of enzyme costs and hydrolysis times would not be possible from the kinetic approach alone.

4.3.7 Economic evaluation of the commercial β-galactosidase preparations.

The costs of the commercial preparations are given in Table 4.10. These costs were supplied by the enzyme manufacturers as their best price FOB at the point of dispatch. These costs were incorporated into the comparison, replacing the amount of enzyme required to achieve 80% hydrolysis by the cost of this amount, as shown in Figures 4.27 and 4.28.

The use of neutral lactases for the hydrolysis of potassium-neutralized acid whey may be the most economical approach. However, if the final product was to be used as the base for a lactose hydrolyzed whey beverage, further demineralization would be necessary to remove the salty and metallic flavours imparted by the potassium added. The need for the extra demineralization equipment would most likely made this alternative unattractive for industrial processors. Enzymes E, D, or C would be a better option if the overall expenses added with the inclusion of a demineralization step are higher than the differential cost of the acid enzymes.

Although enzyme C had the highest activity, this preparation was also the one with the highest cost, and

Table 4.10. Cost of the commercial enzyme preparations.

ENZYME	COST OF ENZYME PREPARATION ($CAN/Kg)[1].
A	36
B	45
C	175
D	138
E	70
F	144

[1] Rate of conversion= 0.80$US/1.00$CAN

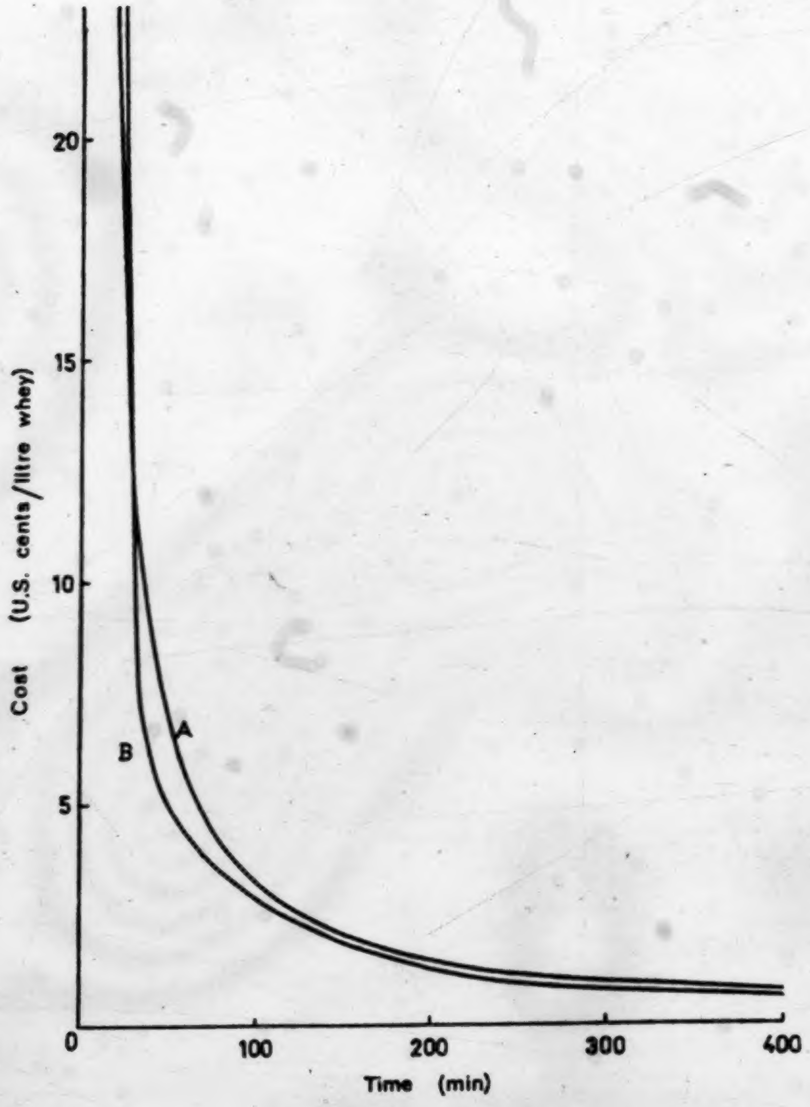

Figure 4.27. Enzyme cost/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using neutral lactases.

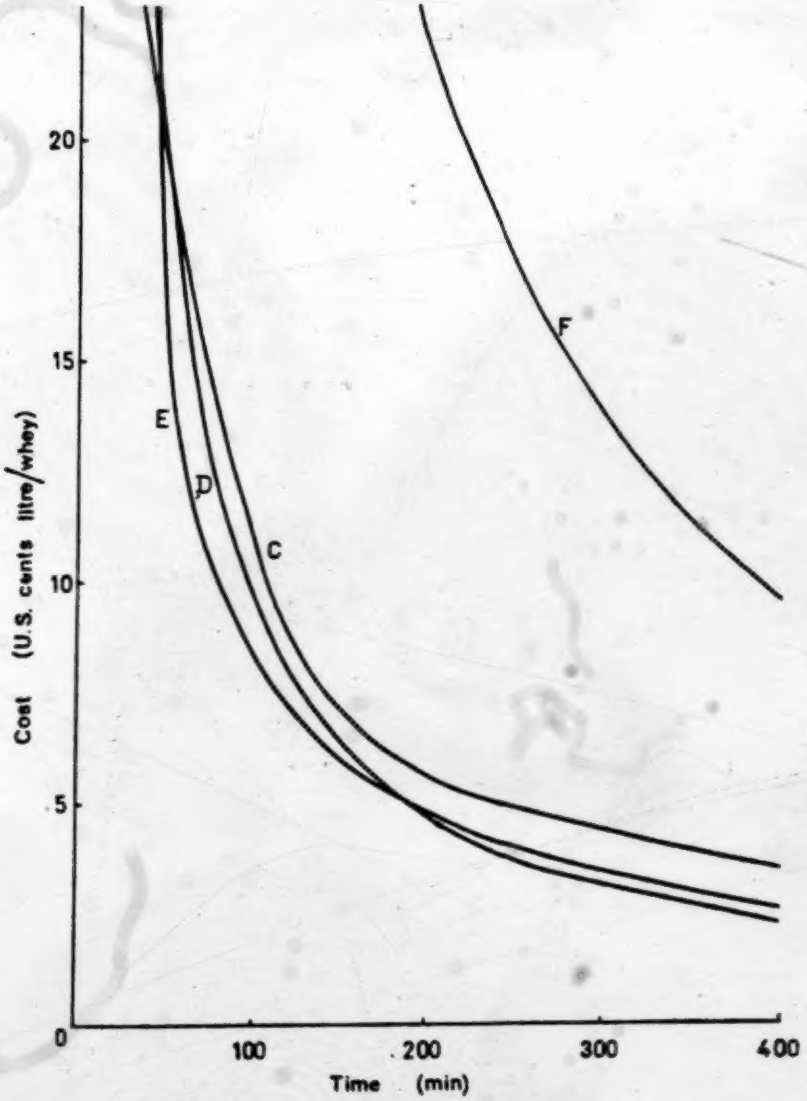

Figure 4.28. Enzyme cost/hydrolysis time combinations required to obtain an 80% lactose hydrolysis in whey using acid lactases.

enzymes E and D thus become a better choice. Enzyme E would be the best choice for the manufacture of a lactose hydrolyzed whey-based beverage if the hydrolysis times desired during the process were in the order of 180 minutes or shorter, while enzyme D would be the best for longer hydrolysis times.

4.3.8 Remarks.

Efficient and not prohibitively expensive ways of hydrolyzing lactose in whey are now available. Depending on the enzyme cost/hydrolysis time compromise considered to be the most appropriate, the cost of the hydrolysis step in the manufacture of a whey-based drink would be in the order of 3-4 cents per litre of hydrolyzed whey (Figure 4.28).

Since the manufacture of most commercial enzyme preparations is at an experimental stage at present, the relative effectiveness of the preparations when compared against each other should be expected to change as the biotechnology improves and the costs of the preparations decrease. Repeated experiments should be carried out by those interested in utilizing this promising new technique in actual industrial conditions.

4.4 Sensory evaluation of a prototype whey drink.

4.4.1 Introduction.

Prototype products were prepared applying the results obtained from the protein precipitation and the lactose hydrolysis experiments. Several drinks were made using either one of the three enzymes found to be the best choices for the hydrolysis of lactose in these products, and adjusting the pH of the whey below the critical point where the protein precipitation can be avoided.

4.4.2 Evaluation of the lactose-hydrolyzed drinks produced with three β-galactosidase enzymes.

Apart from having a good hydrolytic activity, a β-galactosidase enzyme used for the hydrolysis of lactose in the product should not impart any undesirable sensory characteristics to the whey drink.

Triangle tests were used to determine if there were detectable differences in bitterness between the prototype drink made with sugar and three drinks made using the three best acid enzymes (C, D or E). The panelists were asked for bitterness because this defect has apparently been reported to be a problem when using β-galactosidase preparations that may be contaminated with proteolytic enzymes (Jelen, personal communication).

Selected panelists were unable to identify differences in bitterness among any of the samples (Table 4.11). Only three judgments (out of twelve) correctly identified the odd sample when the enzyme C treated product was tested against

Table 4.11.- Correct identification of odd samples during Triangle tests carried out to establish any possible difference in bitterness between a prototype product and lactose hydrolyzed whey drinks.

Test	Number of correct answers	Probability of a chance decision[4]
P-C[1]	3/12	0.819
P-D[2]	6/12	0.178
P-E[3]	5/12	0.368

[1] prototype against enzyme C treated sample.

[2] prototype against enzyme D treated sample.

[3] prototype against enzyme E treated sample.

[4] from Roessler *et al.*(1978).

the prototype. The number of correct answers was six (out of twelve) with the enzyme D treated sample while only five (out of twelve) identified the odd samples correctly when the enzyme E treated sample was tested. If the number of correct judgments for any of the samples tested had been eight or nine, this would be an indication of a detectable difference at the 5% or 1% significance level, respectively (Larmond, 1977).

4.4.3 Comparison of the prototype drink against a commercial product.

To compare the grapefruit flavoured prototype drink against a whey drink available on the market in Edmonton, a paired-comparison test was carried out. The results of this paired comparison are given in Table 4.12. Of the 30 panelists who tasted the drinks, 23 prefered the commercial sample, indicating a significant preference at the 1% level.

The flavour of the products was a definitive factor of preference expressed by the panelists. The commercial product contains three different fruit juices (pineapple, orange and passion fruit) which give this drink its characteristic flavour. A very careful selection of the flavour for the prototype drink would put this product in a better position against the commercial drink. In any population, there are people who like the sweet flavours and others who prefer acid or tart flavours. The prototype drink would be most likely consumed by those who like sharpness in

Table 4.12.- Comparison of a grapefruit flavoured prototype drink against a commercial product.

Main reason for preference	Sample prefered	
	Commercial product	Prototype
	Number of answers	
Flavour	14	2
Sweetness	7	0
Texture	2	0
Acidity	0	5
Total	23	7

taste.

Those who prefered the prototype drink liked its "tartness" and "tanginess"; some said they would drink it if they were thirsty, and thought the commercial drink was too sweet for their like. This last comment was also expressed by some of those who prefered the commercial sample. A repeated comment was that a drink somewhere between the two samples tested would be the best.

It is important to point out that the prototype drink used for the sensory evaluation was only a very preliminary product that could be greatly improved.

5. CONCLUSIONS AND RECOMMENDATIONS.

5.1 Summary of Research Findings and Conclusions.

The problem of whey utilization is, to a large degree, determined by the possibilities for the utilization of lactose, the largest and most "inconvenient" whey constituent after water. Although many processing technologies are currently available, economic constraints have prevented them from being applied.

Lactose hydrolysis using a soluble β-galactosidase seems to be an efficient and relatively inexpensive alternative for the utilization of whey for the manufacture of new food products. This process does not require very large volumes of whey or a very high capital investment for the acquisition of complex and sophisticated equipment. Equipment usually present on the floor of any dairy plant, such as a temperature-controlled tank with a stirring mechanism, would be all what would be needed to perform this operation. Although a more complete evaluation of the process is required, the running cost would not be expected to be high. As shown by our estimates, the cost of the enzyme would be in the order of only 3-4 cents per litre of hydrolyzed whey, under conditions similar to those described in this work. Since the manufacture of the commercial acid β-galactosidase preparations used is only at an experimental stage at present, the manufacturing costs should decline as the process is improved and the market increases. Also important is the amount of sugar or sweetening agent saved due to the increased sweetness added by the hydrolyzed lactose, and the cost of sugar in local conditions.

The major observation confirmed by the present work is that the use of lactose hydrolysis in the manufacture of a beverage from whole, lactose-hydrolyzed acid whey could be a technologically viable alternative even for small and medium size cottage cheese producers. From the six commercial preparations compared in this study, three of the acid β-galactosidases (those from Enzyme Development Corporation, Miles Laboratories, and Rohm GmbH) were found to be approximately equivalent for the purpose refered. Both their hydrolytic activity in whey and the cost of the commercial preparations, the fundamental parameters used in the comparison, were similar.

In the comparison of the commercial preparations, differences were observed between the two approaches examined for this comparison. The composition of the lactose solutions used for the kinetic essays may have been too different from the one of the actual substrate. This point may illustrate why the assessment of the activity of any of the β-galactosidases should be done in cottage cheese whey, if this would be the substrate to be used in the dairy industry.

Furthermore, some factors that may play a key role in the actual behaviour of the β-galactosidases in whey (like galactose inhibition or the gradual decrease in enzymatic activity occuring during the course of the hydrolysis reaction) can not be taken into account by the kinetic based comparison. A third point may be that, in most of the mathematical expressions derived from the Henri-Michaelis-Menten equation and used in this work, the saturation of the enzyme with substrate is a necessary condition for the accurate prediction of the enzyme activity. As can be noticed from the K_m values obtained, lactose concentration in whey is not high enough for the enzymes to be saturated with lactose.

The use of neutral β-galactosidases for the hydrolysis of KOH-treated acid whey gave good results. However, the use of this hydrolyzed KOH-treated whey is not recommended in the manufacture of a whey-based beverage, because KOH may impart undesirable sensory characteristics to the final product, making it unacceptable for direct human consumption. The additional demineralization step that would be needed also makes this approach unattractive for the small or medium size processors.

Nevertheless, if the whey is to be used for the production of ethanol or methane, the use of a neutral lactase to hydrolyze cottage cheese whey may be a successful and profitable operation. The activity of the neutral β-galactosidases seemed to be strongly influenced by the pH-adjusting procedures used; also worth noting were the different responses to K^+ observed in the two neutral enzymes studied.

The problem of protein precipitation common to heat-treated whey-based beverages could be controlled by adjusting the pH of the drink to below 3.7 prior to thermal processing. Under these conditions, most of the whey proteins were not precipitated after heating at 95°C for 5 minutes. Electrostatic repulsion forces created when the pH is lowered presumably prevented the denatured proteins from aggregating together and precipitating. The retention of the protein solubility in the drink would give a good appearance while maintaining excellent nutritional qualities of the final product.

The thermal behaviour of a whey protein concentrate, and probably of the whey proteins in the whey itself, seems to be dominated by the behaviour of β-lactoglobulin. The denaturation temperatures of the whey protein concentrate and the major isolated whey proteins were pH-dependent, and were also affected by some other whey constituents. Thus, bovine serum albumin was protected against heat-denaturation by fatty acid binding. The denaturation temperature of β-lactoglobulin was increased in the presence of lactose, and a slight further increase was obtained when this sugar was replaced by glucose and galactose (as if β-galactosidase had been used to hydrolyzed lactose in whey). Important observations were made on the influence of calcium on the thermal behaviour of α-lactalbumin; the denaturation temperature, the enthalpy , and the reversibility of the process were all affected by the removal of the calcium with

EDTA.

5.2 Recommendations.

5.2.1 Recommendations for the development of a whey beverage.

As mentioned previously, the prototype product prepared for this study could and should be improved. The following are some steps that may be taken to achieve this improvement: 1) to define the sector of the population towards which the product would be targeted. Perhaps the optimum product for sportsmen would be different from that suitable for children or lactose intolerant adults. 2) To identify the variables that are important for the sensory acceptance of the drink by the target population. Flavour, freshness, sweetness or tanginess may be some of these factors. Their degree of importance should also be determined. 3) To find the right combination of variables needed to get an optimum acceptance.

The next step required after the improvement of the prototype drink would be the completion of the product development stage, coupled with an extensive market research study.

The utmost care should be put into the selection of the flavour. This research should perhaps be done by a team of specialists in flavour development. Probably the flavours more compatible with the whey characteristics would be the citrus fruit flavours, such as grapefruit, lemon or orange. However, other flavours like apple, passion fruit or perhaps almond may also be considered. Most likely, colour will also be added. Both colour and flavour should be acid and heat stable.

Some research will be needed to identify the most suitable acid for the pH adjustment step. Citric acid was used for the prototype drink, but some other acids or mixture of acids (i.e. ascorbic acid) may be worth trying. Although the sensory properties of the drink should be the main criteria used for the selection of the acidifying agent, cost may also be important.

The selection of a sweetening agent may be another area of improvement. Although sugar is perhaps the least expensive and most traditional sweetening ingredient, maybe the use of some artificial sweeteners, particularly aspartame, could have a strong impact on the acceptability of the product by the consumer.

The hydrolysis of lactose in the preparation of a whey-based beverage would make this product available to those individuals who have lactose intolerance problems and therefore cannot drink milk. Since none of the acid enzymes tested affected the sensory attributes of the prototype product prepared, the choice of the β-galactosidase should depend strictly on economic considerations. The length of the hydrolysis would depend on the amount of β-galactosidase used, which would be determined by the enzyme cost/hydrolysis time compromise considered to be most appropriate. The cost/ hydrolysis time combination plots prepared from the progress curves would be also very useful when selecting the hydrolysis conditions most suitable for the integration of the drink manufacture into the whole cheese making operation. The industrial scale-up of the process, along with a more detailed economic evaluation, would be necessary before this point.

By combining the technical information disclosed in the present work with that obtained from the marketing research, the best possible product in its class could be developed.

It should be kept in mind that all the information given in the present work was obtained under laboratory conditions and needs to be tested at least on a pilot plant scale before further recommendations can be made for the application of this work to an actual industrial situation.

5.2.2 Recommendations for future research work.

The enzyme market is continuously expanding, and new β-galactosidase preparations will probably be introduced in the near future. Since these new preparations are expected to bring improvements in the product quality and decrease the cost of lactose hydrolysis technology, they should be continuously evaluated.

Perhaps the kinetic-based approach used in the first part of the evaluation of the commercial preparations would give better results if the kinetic parameters of the enzymes are determined under conditions as similar as possible to the actual industrial substrate. The use of reconstituted whey concentrate and diluted whey may be the solution to cover the whole lactose concentration range required to do the Michaelis-Menten experiments.

The effectiveness of the neutral β-galactosidases in KOH-treated sweet whey should be compared against that in KOH-treated acid whey, in order to establish the actual benefits obtained when acid whey is hydrolyzed with a neutral enzyme, after neutralization with KOH.

Galactose inhibition and oligosaccharide formation during the hydrolysis may be interesting areas for continuation of basic research with the soluble enzymes.

In the protein denaturation area, it would be worthwhile to continue the model system approach to elucidate the thermal behaviour of proteins in whey. The influence of pH and non-protein whey constituents on the interactions occuring between β-lactoglobulin, α-lactalbumin and bovine serum albumin should be investigated using model systems containing two or three proteins. The importance of disulphide exchange reactions should be also explored.

The study of α-lactalbumin brought about many questions that remain to be answered. The mechanisms of acid and heat denaturation, and the role of calcium on the reversibility of the denaturation process may require a much more complete study than was possible within the present context of this applied research project.

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